THE ANTI-GLOBULIN (COOMBS) TEST IN LABORATORY PRACTICE

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DEDICATION

The Book is dedicated to Miss Elizabeth W (Jan) Ikin B Sc —a kindly person to whom so many blood group workers owe so much

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INTRODUCTION

THE anti globulin reaction, or Coombs' test, has played a major part in the development of our knowledge of blood groups in man and animals It is an essential technique in the forecasting and diagnosis of hæmolytic disease of the newborn in various species, and through its use the transfusion of blood from donor to patient has become a safer procedure The bacteriologist finds it a useful technique for the detection of bacterial antibodies, the forensic scientist can apply it in the detection of crime, the hæmatologist uses the test routinely in the investigation of hæmolytic anæmias, for the study of antibodies to various cellular elements of the blood and he may possibly find it helpful in the study of certain plasma fractions, finally the experimental immunologist applies it to study antigen-antibody reactions

Published descriptions of the anti globulin test as originally devised, and the more recent developments, modifications and extensions of the technique are widely scattered in various journals, making it exceedingly difficult for the student to grasp the underlying principles and to apply them correctly in practice

This book is an attempt to provide, for those interested in medicine bacteriology, hæmatology, blood transfusion and the veterinary and forensic sciences an explanation of the fundamentals of the anti globulin test with suggestions for further reading which they may find helpful in the application of the test to their own particular problems

CHAPTER I

ANTIGENS AND ANTIBODIES

BEFORE embarking on the main theme of this book, it is proposed to define for the reader some of the terms in common use in the field of immunology.

The essential part of all the tests to be described later is the antigen antibody reaction. But what are antigens and antibodies?

An *antigen* is any substance which, when introduced into the body of an animal to which it is foreign, is capable of stimulating the host animal to produce a specific anti-substance or antibody. Antigens may be protein in nature and some carbohydrates are also antigenic. The animal which has been provoked, by the introduction of a foreign antigen, into producing an antibody directed specifically against that antigen is said to have been *immunised*.

An *antibody* is a substance found in the blood plasma or serum which reacts with cells, or substances in solution, containing the antigen for which it is specific. Such antibodies are probably formed in the body tissues and sometimes their presence can be demonstrated in other body fluids such as colostrum and peritoneal exudates. They are part of the globulin fraction.

The most easily demonstrated *antigen antibody reactions* are those used in blood grouping. Serum containing antibody of known specificity is mixed with a saline suspension of red blood cells. If these cells then form clumps or agglutinates they are assumed to carry the equivalent antigen, if they do not agglutinate they are considered to lack the antigen. Conversely, by mixing a serum which is to be investigated with a series of suspensions of red cells of known antigen content it is possible, by noting

reaction regardless of the composition of the fluid in which the antigen is suspended, or the degree of magnification employed to discover it The presence of these crypt-agglutinoids can be disclosed only indirectly by the antiglobulin technique

The Mechanism of the Antigen-Antibody Reaction

The forces underlying the antigen antibody reaction are still a matter for conjecture The most practical hypothesis envisages it as a two stage response in which the stages are not distinct for it is possible that the second stage starts before the first is complete

In the first stage the antibody rapidly forms a chemical combination with the antigen although it produces no outward evidence that such a combination has occurred The second stage, which progresses more slowly, is the phase of observable reaction in which agglutination or precipitation becomes obvious It is probable that the second stage is a physical response depending upon, or modified by, such factors as the composition of the medium in which the reaction has occurred, the relative quantities and reaction potential of antibody and antigen, and the presence or absence of complement Antigen antibody reactions are not all of the same type and they do not all require the operation of the same factors As an example, for the detection of most lysins complement must be present, but it is not an essential ingredient in the demonstration of agglutinins

Following this hypothesis it would appear that incomplete antibodies are those which participate in the specific combination of stage one, but are unable to complete stage two so long as the surrounding medium is of small molecular size How can we know that stage one has in fact occurred ? When the antigen is part of a red blood cell the fact that it

the occurrence of agglutination, to determine whether the serum contains an antibody and what its specificity is

CLASSIFICATIONS OF ANTIBODIES

Antibodies can be divided into a number of categories They may be classified according to the manner in which they react with the antigen *in vitro* An antibody which precipitates the antigen is called a *precipitin*, one which dissolves the antigen is a *lysin*, while an antibody which will produce clumping of cells carrying the antigen is an *agglutinin* The antigens corresponding to these varieties of antibody are respectively designated precipitinogens, lysinogens and agglutinogens The reactions between these antibodies and antigens can be seen by the unaided eye or with a magnifying lens or microscope

A slightly different classification divides antibodies into two broad groups based upon their ability to interact with the antigen suspended in isotonic saline solution *Complete antibodies* (sometimes called *bivalent* antibodies) are those which cause an obvious alteration such as agglutination, precipitation or lysis of the appropriate antigen in saline medium The *incomplete* (or so called *monovalent*) *antibodies* are unable to produce such visible evidence of a reaction with their corresponding antigen in saline and to demonstrate their presence additional procedures are required

Two types of incomplete antibody are recognised—the agglutinoids and the cryptagglutinoids An *agglutinoid* is an antibody which exhibits no visible effect on the antigen unless the reaction takes place in a protein medium such as bovine albumin or in certain macro molecular solutions such as dextran or until the antigen has first been modified by enzyme action Agglutinoids are sometimes called albumin antibodies *Cryptagglutinoids*, on the other hand, are antibodies which fail to bring about any observable

Not all incomplete antibodies have this capacity to block, wholly or partially, the corresponding antigen

The interactions between red cell antigens and complete and incomplete antibodies are more easily understood when represented diagrammatically The first illustration (fig 1) shows, in diagrammatic form, a red cell with blood group antigen receptors on its surface and alongside it are two

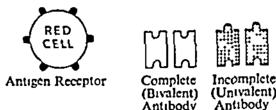


FIG 1

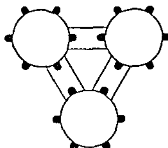


FIG 2 Cells agglutinated by complete antibody

complete and two incomplete antibody molecules The agglutination of red cells by complete antibody is visualised as a uniting of the cells (fig 2) by bivalent antibody bonds In contrast, incomplete antibody attaches itself to the antigen (fig 3) but being monovalent, fails to produce agglutination *unless an extra something is provided by bovine albumin which links (fig 4) the coated cells together*

It must be emphasised that the exact nature of the differences between complete and incomplete antibodies, and the mechanism of the antigen antibody reaction, are not yet completely understood The diagrams depict symbolically what is thought to take place but they do not explain

has combined with its corresponding antibody can be demonstrated in at least two ways.

Firstly, let us suppose that some red cells possessing the antigen X (X positive cells) are suspended in saline and are then mixed with serum containing an incomplete anti X antibody. They do not agglutinate in spite of their contact with the corresponding antibody. If however, the supernatant serum and saline are removed from the sedimented cells and an equal volume of a 20% bovine albumin solution is substituted, it will be found that, provided the time allowed has been sufficient and the temperature at which the test is performed is suitable, agglutination of the cells will occur. This agglutination is not due to the action of the bovine albumin alone since X-positive cells which have not been exposed to anti X do not spontaneously agglutinate when suspended in the albumin. Therefore the antibody—incomplete anti X—must have combined with red cells. That the reaction is specific is shown by the fact that the incomplete anti-X will not combine with red cells other than those carrying the X antigen. This *albumin replacement* technique [1] is a particularly useful method for detecting the Rhesus (Rh or D) antigen in the red blood cells in man. Only incomplete antibodies of the agglutinoid type will bring about this agglutination reaction, cryptagglutinoids fail to clump the cells even in the presence of bovine albumin.

A second proof that stage one has occurred is provided by the *blocking test* of Wiener (1944) [2]. A saline suspension of X positive cells is mixed with an incomplete anti X serum. If, after a time the cells are washed free from incomplete anti X and are then mixed with a complete anti X serum the normally expected agglutination will either not occur or if it does, the degree of agglutination will be much less than that shown by X positive cells not previously exposed to incomplete anti X. The incomplete antibody has blocked, inhibited or prevented the action of the complete antibody

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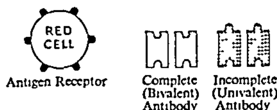


FIG 1

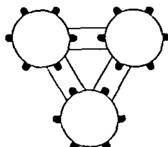


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It must be emphasised that the exact nature of the differences between complete and incomplete antibodies, and the mechanism of the antigen antibody reaction, are not yet completely understood The diagrams depict symbolically what is thought to take place but they do not explain

all the types of reactions that have been observed For instance, it has been shown that incomplete blood group antibodies can be made to agglutinate their appropriate red cells suspended in saline provided that these cells carry more than the usual amount of antigen,[3] or provided that the cell surface has been modified, increasing the number of antigen receptors, by partial digestion by enzymes[4] It may be that ability or otherwise of an incomplete antibody to agglutinate red cells in saline is governed not by the number

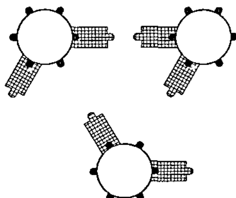


FIG 3 Cells coated by incomplete anti body (not agglutinated)

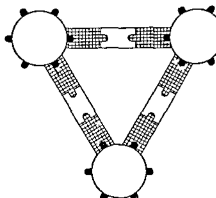


FIG 4 Coated cells agglutinated by addition of bovine albumin

of combining sites on the antibody molecule but, rather by the number of antigen receptors and hence by the amount of antigen available on the cell surface

The foregoing explanations have dealt mainly with antigen antibody reactions as seen in blood group serology, but it has been shown that similar incomplete antibodies to bacterial antigens exist The two main points to be borne in mind are (1) that some antibodies will give rise to no visibly detectable reaction if the antigen is suspended in saline only, and (2) that no negative reaction can be accepted unless the possible presence of incomplete antibodies—agglutinoids and cryptagglutinoids—has been excluded

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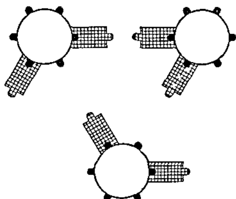


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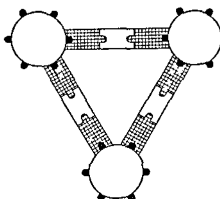


FIG 4 Coated cells agglutinated by the addition of bovine albumin

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CHAPTER II

THE SEROLOGICAL BASIS OF THE ANTI GLOBULIN TEST

SPECIES DIFFERENCES

EACH species of animal has, in its blood cells, tissues or body fluids, its own *species specific substances* The differences between these substances cannot always be detected by chemical tests although they can be shown to be serologically distinct By injection of red cells or serum obtained from one animal into another animal of a separate species an *anti species antibody* can be provoked which may be an agglutinin, precipitin or lysin and which may be complete or incomplete in character Since all anti globulin sera are anti species antibodies it is essential for the user to be aware of the possibilities of cross reactions and of the various differences and similarities between species

The injection of human red cells into a rabbit will lead to the development by the rabbit of anti man antibodies Although such antibodies can be made specific (able to react only with the precise antigen which stimulated their production) it is often found that they will also react with red cells and other tissue cells from related species They combine most strongly with the antigen employed to provoke them (the *homologous antigen*) but they may also react to a lesser degree with antigens obtained from a related species of animal (*heterologous antigens*) For instance, fowls, immunised with rabbit serum develop an antibody which reacts with both rabbit and hare serum and yet a hare, immunised with rabbit serum, will produce an antibody capable of reacting with rabbit serum but not with hare serum For more information on the preparation of anti species antibodies the work of Nuttall (1904)[1] is well worth

consulting For an understanding of serological specificity the student is referred to the monumental work of Landsteiner (1945)[2]

Anti Globulin Antibodies

When human serum is injected into a rabbit the rabbit responds by making an anti-species antibody which will agglutinate all human red cells, but this may not be the only antibody which has been stimulated This first antibody can be removed by mixing the rabbit's serum with well-washed human red cells The anti human species antibody becomes attached to the red cells and can be discarded with them, leaving in the rabbit serum a different antibody The second antibody is an *anti globulin* provoked by the antigenic action of the globulins which were present in the human serum used initially to immunise the rabbit

The anti globulin antibody is also a species specific antibody—in this case the rabbit produced an anti-human globulin It will not agglutinate normal human red cells but it will react with any globulins derived from a human source such as whole serum and certain body tissues As mentioned previously, antibodies are themselves globulins and therefore the anti globulin antibody may be thought of as an anti-antibody antibody ! In a mixture of two sera, one containing antibody and the other containing the correct species anti globulin antibody, the reaction could be manifest as a precipitate

There is, however, a more usual and more useful way of using an anti globulin serum, which forms the basis of the Coombs test Let us take as an example the case of a patient who has received a blood transfusion and who, as a result, has developed an incomplete antibody to the blood-group antigen called " Rh " The incomplete anti-Rh serum from this patient will not agglutinate Rh positive cells suspended in saline, but the antibody will become attached

to the cells, giving them a coating of human globulin, there may be also some excess of uncombined antibody in the supernatant saline To rid the serum saline mixture of unwanted globulins which would readily combine with anti globulin antibody and spoil the test, we must "wash" these coated cells This we do by (1) centrifuging the mixture, (2) removing the supernatant serum saline solution (3) replacing this by a large volume of fresh saline, (4) mixing the cells thoroughly, and (5) re centrifuging For

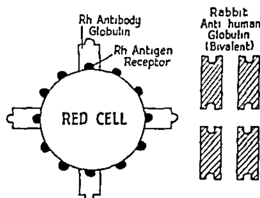


FIG 5

efficient washing this cycle has to be repeated several times and the end product is a saline suspension of cells in which the only globulin present is the antibody coating the cells, i e specifically combined with the antigen (One word of warning should be interpolated here—cell washing is usually performed at room temperature or occasionally at 37 C Higher temperature should not be used since this may result in removal of the antibody from the cells)

We now take a rabbit anti human globulin serum—prepared from the serum of a rabbit which had received injections of human serum and from which the anti species antibody has been absorbed If we mix this rabbit serum with the washed, coated red cells the anti globulin antibody reacts with the globulins on the cell surfaces, causing the

cells to clump together as if the agglutination were due to the action of the anti globulin alone We know this appearance to be false because the specific rabbit anti human globulin antibody will not cause agglutination of human red cells unless they have first been exposed to the action of a specific incomplete antibody

Again, pictorial representation of the supposed mechanism may be helpful The first diagram (fig 5) shows a red cell

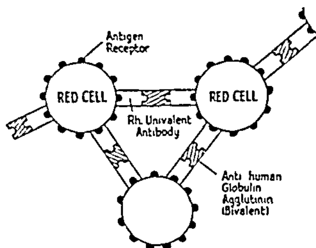


FIG 6

coated with incomplete antibody, together with four molecules of rabbit anti human globulin antibody The second diagram (fig 6) illustrates the attachment of red cells to each other by the bonding effect of the anti globulin reacting with the globulins on the cells' surface

THE ROLE OF COMPLEMENT

The work of Hackett (1950)[3] has shown that, so far as anti human globulin human globulin reactions are concerned, the addition of guinea pig complement neither enhances nor inhibits the reaction On the other hand, it has also been found that certain incomplete antibodies to red cell antigens in man, e g anti Kidd, anti Lewis, will coat red cells in the

absence of complement, or complement like substances, but this coating will not be detected by the anti globulin reagent[4] However, if to the sensitised cells fresh human serum is added and then the cells are rewashed, a positive anti globulin reaction can be obtained

NOMENCLATURE

When referring to anti globulin antibodies it is usual to describe them in such a way as to indicate both the animal in which the antibody was produced and the specificity Throughout this chapter we have mentioned "rabbit anti human globulin serum" In an alternative terminology the name of the immunised animal is placed last Our example then becomes ' anti human globulin rabbit serum

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CHAPTER III

HISTORY AND NOMENCLATURE

Many people refer to the anti-globulin test as the Coombs' test and believe that it was discovered by Dr R R A Coombs of Cambridge University in 1945 In fact, the principle of the test was first employed in 1908 by an Italian immunologist, Carlo Moreschi (1908)[1, 2]

Moreschi diluted specific anti sera to red cells and bacteria until the sera no longer caused agglutination He then thoroughly washed the unagglutinated cells and exposed them to anti species sera and obtained agglutination The anti species sera were specific for the animals which provided the original sera which, diluted, failed to cause agglutination Interpreting Moreschi's observations it would appear that on diluting the sera he diluted out the complete antibodies, but left higher-titred incomplete antibodies capable of coating the bacteria and the red cells This coating would be a globulin, and the anti-species sera contained anti globulins which caused the agglutination of the sensitised cells

Unfortunately, Moreschi's technique was not applied in routine serology for the next 37 years and it was only in 1945, when the problem arose of finding new methods with which to detect the recently discovered Rhesus antibodies, that Coombs, Mourant and Race (1945),[3] unaware of Moreschi s earlier work, devised this method Dr Coombs (1954)[4] in a lecture delivered in Rome, paid tribute to Moreschi for his discoveries

Without wishing in the least to detract from Dr Coombs' well deserved distinction, it becomes obvious that the term 'Coombs' test" ignores the contributions of Moreschi and of Mourant and Race Dr Coombs himself calls the technique "the anti globulin sensitisation test" To

include the word "sensitisation" makes the title rather cumbersome for routine use, but it is an advantage to omit the reference to species (as in "anti human globulin serum"), thereby giving a general description of the method irrespective of the anti species globulins used

The term "*anti globulin test*" indicates the potential breadth of application of the technique and is to be preferred However, in describing the method, especially when cells and sera from different animals are used it is essential to specify the species from which the antigen was obtained and the animal in which the anti globulin was developed e g goat anti rabbit globulin or anti rabbit globulin goat serum

Hill, Haberman and Jones (1948)[5] devised the name *Developing Test*" for the anti globulin technique It is an apt descriptive title since the anti globulin serum "develops" the agglutination of coated cells in a way analogous to that in which various chemicals act upon a photographic plate There is no indication, however, of what is being developed nor of which reagents are being used

The Direct and Indirect Anti Globulin Tests

The anti globulin test can be used in two ways Where there is reason to suppose that red cells may have been coated by antibody, the addition of anti globulin serum will show by the subsequent production of cell clumping or by a lack of agglutination whether or not the cells have been sensitised The name coined by Coombs Mourant and Race (1946)[6] for this technique is the *Direct anti-globulin test*

The *Indirect anti globulin test* is used in the investigation of any serum which it is thought may contain incomplete antibody The first step is to mix the unknown serum with red cells and to allow time for the antibody if present to become attached to the cells If the cells are then thoroughly washed and examined for sensitisation by the

Direct anti globulin method, the development of agglutinates will indicate that an incomplete antibody, capable of coating the cells, was present in the serum which was being investigated

A H G

"Human nature being what it is, it was not surprising that those working with anti human globulin serum in 1946 soon found this title too burdensome and substituted for it the abbreviation A H G" It was unfortunate, therefore, that some hæmatologists about the same time decided to use the initials A H G for an entirely different substance, anti hæmophilic globulin, a fraction of plasma employed to combat the deficiency present in hæmophilia

Considerable argument can be adduced for and against the use of the letters A H G to signify the anti hæmophilic globulin but it is of conspicous practical importance that, in any laboratory handling both the anti hæmophilic factor and anti human globulin serum, steps should be taken to avoid confusion between them The distinction can be satisfactorily maintained by calling the former A H F and the latter A H G

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these Eyquem (1956)[4] and Goodman and co-workers (1957)[5] used, respectively, donkeys and chickens '

The choice of animal may be governed by the volume of the yield required The amount obtainable from a goat will be ten times that which can be procured from a rabbit Nevertheless, when small quantities only are needed, or when several reagents from different animals are required, the rabbit is an ideal animal

Within a species, not all animals are equally good antibody producers The varieties of rabbits known, in the United Kingdom, as Chinchilla, Flemish, Lop and Belgian Hare are generally more satisfactory than Rex or Dutch rabbits When a strain of rabbits is discovered to be particularly competent in producing antibodies their offspring, too, will generally be found to be good antibody producers This suggests that the ability to develop antibodies readily may be genetically determined and, therefore, breeding from such stock is recommended

Experience with goats may be similar Dunsford (unpublished observation), using five goats of no particular breed, has not yet failed to obtain a satisfactory serum, but Proom (1957)[6] has been less fortunate

A full protein diet supplemented with vitamins, etc, is recommended for rabbits On the other hand, goats appear to be capable of antibody production on any diet—even including a laboratory coat or the morning newspaper if available[1] Laboratories maintaining animal houses will find excellent guidance in the U F A W Handbook, *On the Care and Management of Laboratory Animals*, published by the Universities' Federation for Animal Welfare, London, Second Edition, 1957 In countries where myxomatosis is prevalent precautions should be taken to reduce the chances of the rabbits becoming infected They can also be protected by vaccination (a suitable vaccine is prepared by Burroughs Wellcome Ltd , London)

CHAPTER IV

THE PRODUCTION OF ANTI-GLOBULIN SERA

THE serum of an animal immunised by injection of serum from another animal may be found to contain two or more antibodies —

1 The anti species antibody which will agglutinate or hæmolyse red cells of all members of the species from which the immunising antigen was derived,
2 The anti species globulin antibody—the reagent required for the anti globulin test,
3 An hetero antibody, which may be naturally occurring (present in the unimmunised animal) or immune For instance, rabbits are commonly found to have in their sera a naturally occurring antibody active against human group A red cells

Before our anti globulin serum can be used it must be freed from the unwanted anti species and hetero antibodies Although similar procedures may be used to make, for example rabbit anti fowl globulin serum in the interests of simplicity the method of preparing anti human globulin serum only will be described

CHOICE OF ANIMAL

The rabbit being comparatively small and easy to handle, is the animal most commonly used for the production of anti human globulin serum However it is quite possible to immunise other species Hill and Haberman (1954)[1] and Dunsford and Bowley (1957)[2] have successfully immunised goats Stratton (1956)[3] had success with sheep and also experimented with a variety of other species including hens and horses, though he does not comment on his results with

suffering from acquired hæmolytic anæmia In this disease the patient's red cells are prematurely destroyed within his blood vessels and his serum may be found to contain antibodies against his own red cells These antibodies may be of two types—those which agglutinate red cells more readily at 37° C (warm type antibodies) and those which react more strongly at room temperature or below (cold antibodies)

Komninos and Aksoy (1955)[14] have used as their antigen coated red cells from cases of acquired hæmolytic anæmia, and claim also to have produced potent anti-globulin sera by the injection of antibody eluted from such cells

Pisciotto and Hinz (1956)[15] have used sera obtained from patients with acquired hæmolytic anæmia and assert that the anti-globulin serum resulting from immunisation by a serum containing warm type antibody differs in specificity from the anti globulin serum evoked by a cold type antibody This may be explained to some extent by the findings of Christensen and Dacie (1957)[16] who, by electrophoresis, showed that although sera from "warm antibody" hæmolytic anæmias have no characteristic electrophoretic pattern, sera from cold antibody" hæmolytic anæmias have a peak in the γ_1 region It suggests, too, that even better reagents may be prepared by using as antigens, sera from patients with abnormal globulins such as are found, for instance, in multiple myeloma

✓Sera from people whose blood groups are A, B or AB should not be used as antigen in the production of anti-globulin sera since they contain A and/or B blood group specific substances in solution which are antigenic and would cause the rabbit or goat to produce anti A and/or anti-B antibodies in addition to the required anti human globulin To avoid the necessity for removing such additional antibodies it is the custom to use a pool of sera obtained from

Choice of Antigen

In the search for the ideal antigen for the production of anti globulin serum many materials have been tried, including serum, plasma, gamma globulins, coated red cells and antibodies eluted from coated cells

Coombs, Mourant and Race (1946)[7] in their original experiments used either whole human serum or human gamma globulin and found them equally efficacious, though whole serum was the easier reagent to prepare The authors of the M R C Memorandum No 27 (1952)[8] preferred gamma globulin Hill and Haberman (1954)[1] favoured whole serum Wootton (1950)[9] preferred gamma globulin while Singh Ramakrishman and Prakash (1952)[10] found plasma to be as effective as serum

Kidd (1957)[11] has used gamma globulins prepared by Kendall s method but since some anti red-cell antibodies are non gamma globulins he later modified his immunising schedule by giving his animals alternate courses of whole serum and gamma globulins in order to guard against producing a serum with too narrow an anti globulin spectrum'

Milgrom Luszczynski and Dubiski (1956)[13] employed, as antigen globulin which had been denatured on the surface of red cells as a result of an antigen antibody reaction Rabbit red cells were agglutinated by human serum which had previously been heated at 56 C to inactivate the complement After having washed the rabbit red cells free of excess human serum the agglutinated cells were then injected into a rabbit Bi weekly injections for 6 weeks produced a satisfactory reagent containing very low titre anti human species antibodies requiring little absorption and the sera were free from zoning

A slightly different approach has been used by those authors who have obtained cells or serum from patients

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obtain a better or quicker response in an animal which has been made slightly febrile by the injection of a "foreign" protein Kidd (1957)[11] mixes dried tubercle bacilli with gamma globulin and emulsifies the mixture with Arlacel A (Mannide mono-oleate) With this reagent good anti globulin sera are produced with low-titre anti human antibodies

The Immunising Injections

The routes by which the antigen may be introduced are (*a*) intramuscular, (*b*) intraperitoneal, or (*c*) intravenous As a rule, goats and sheep receive whole serum or gamma-globulin solution by injection into the scapular or gluteal muscles When dealing with rabbits one or more routes may be used and it has been found that greater success in achieving immunisation will follow the use of all three routes for the initial course Rabbits are rather susceptible to anaphylactic shock when the material is introduced intravenously To reduce this risk an intraperitoneal injection should be given less than 24 hours before all intravenous injections

The dosage of gamma globulin required does not appear to be critical The course recommended in the M R C Memorandum No 27 (1952)[8] employs Proom's alum-precipitated gamma globulin (1943)[17] Five ml of the solution are injected into each buttock, this injection is repeated after 14 days and 10 14 days later the animal is bled It is claimed that there is less risk of anaphylaxis with this technique

When whole serum is being used as antigen 0 5 1 ml of undiluted serum (or of 50 per cent serum diluted with saline) is injected intraperitoneally This is followed within 24 hours by an intravenous injection, into the external peripheral auricular vein, of 0 5 ml of 50 per cent serum diluted with saline Thereafter, for the next 3 4 days, daily injections

several group O persons The serum pool need not be fresh, but it should be sterile ✓

The Preparation of Gamma Globulin
(Proom's (1943)[17] method)

1 Dilute 25 ml of pooled group O serum with 80 ml of distilled water
2 Add 90 ml of 10 per cent aqueous solution of potassium alum (K Al(SO_4) $12H_2O$)
3 *Adjust the pH of the mixture to 6 5 by adding 5N sodium* hydroxide The *p*H can be determined by placing a drop of the mixture on a tile and mixing with it a drop of Universal Indicator (obtainable from British Drug House London) Since the *p*H change is sudden the caustic soda should be added carefully drop by drop
4 Centrifuge the mixture and remove the supernatant fluid
5 Wash the precipitates twice with 200 ml of a 1 10 000 saline solution of Merthiolate (Eli Lilly & Co)
6 Make the washed precipitate up to a volume of 100 ml by adding a further saline solution of 1 10 000 Merthiolate

This suspension can then be stored for at least 14 days at 4 C

Repeated injections of alum precipitated globulins are apt to produce sterile abscesses in the muscles and sometimes a paralysis of the hind quarters For this reason it is recommended that although precipitated globulins may be used initially, once the animal has become immunised pooled whole serum should be used for further boosting of the antibody

Satisfactory anti globulin sera can be prepared without the use of an adjuvant though some workers feel that they

obtain a better or quicker response in an animal which has been made slightly febrile by the injection of a "foreign" protein Kidd (1957)[11] mixes dried tubercle bacilli with gamma globulin and emulsifies the mixture with Arlacel A (Mannide mono-oleate) With this reagent good anti-globulin sera are produced with low titre anti human antibodies

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of 1 ml serum are given either intramuscularly or intra venously

Slavin (1950)[18] has advocated a method of immunisation by slow absorption A mixture is made of 1 ml sterile group O serum with 4 ml of a sterile 4 per cent sodium alginate solution ("Calgitex" obtainable from Medical Alginates Ltd, Wadsworth Road, Perivale Middlesex) The mixture is injected intraperitoneally into a rabbit and immediately afterwards a second injection, of 2 5 ml of a sterile 1 per cent aqueous solution of calcium chloride, is *made from a separate syringe into the same site* The calcium chloride reacts with the sodium alginate to form a rubbery lump from which the serum is liberated slowly as the mass breaks down

Once the animal has become immunised it should be left as long as possible before booster injections are given Stratton[19] observed that the longer the animal was left the greater was the titre of the antibody after boosting and acting on his suggestion to obtain the best results the next course of injections should be given only after three months or more However, the antibody evoked by the initial course is likely to be mainly anti human gamma globulin and since other anti globulin antibodies may be required repeated courses may be necessary before the animal is given its rest period

The 'booster course' recommended for an immunised animal is

1 One ml sterile pooled O serum is injected intraperitoneally
2 Within the next 24 hours 0 5 ml of 50 per cent sterile pooled O serum in saline is injected intravenously
3 On the third and fourth day 1 ml sterile pooled O serum is given either intravenously or intramuscularly
4 10 14 days later bleed the animal

BLEEDING THE ANIMAL

The best time to bleed the animal is 10 14 days after the completion of the course of injections though small amounts may be repeatedly removed between the 10th and 30th days Only a small sample for examination should be taken from an animal which has completed its initial course of immunising injections and tests on this this will show whether further bleeding would be justified

When dealing with goats and sheep the blood is collected from the external jugular vein after the hair or wool has been clipped from the neck The help of an assistant, who can manœuvre the beast into a suitable position and hold it firmly there during the operation, is invaluable The animal is backed into a corner, the assistant stands astride its back, gripping its flanks firmly with his knees, he then raises the animal s head upwards and slightly backwards and holds it there The operator locates the line of the external jugular with his finger tips, cleanses the skin over it with spirit and inserts a long, large bore sterile needle (20 cm by 3 mm external diameter) in an upward direction The blood is allowed to run straight from the needle into a sterile collecting bottle When 400-500 ml have been collected the needle is withdrawn from the vein and the bleeding is stopped by firm digital pressure over the site for 4 5 minutes Meanwhile a sterile rubber wad and metal cap have been screwed on to the bottle of blood which is placed in the incubator at 37° C until clotting has taken place As much serum as possible is aseptically removed and then the clot is refrigerated at 4° C overnight On the next day the remainder of the serum is separated from the clot The second lot of serum is sometimes rather hæmolysed

Rabbits may be bled by heart puncture or by nicking the external auricular vein In the hands of those who are not

experts cardiac puncture seems rather brutal and, since a satisfactory yield can be obtained from the ear vein, this latter method is usually preferred The rabbit is immobilised either in a suitably designed box, or in the hands of a trained assistant The hair of the ear is shaved and the bare area over the vein is cleaned with alcohol A small smear of petroleum jelly round the site and over the edge of the ear will prevent the blood from clotting before it has been collected The vein may be punctured with a small needle or a small longitudinal slit may be made with a scalpel and the blood is allowed to drip into a small bottle In this way 40 ml of blood can be obtained Bleeding is stopped by firm pressure if clotting has not already taken place The blood is allowed to clot at 37° C, is kept at 4 C overnight and the serum is separated from the clot on the following day

Inactivation of the Serum

Immediately the serum has been separated it must be heated to 56° C and maintained at that temperature for not less than 30 minutes in order to inactivate the complement, otherwise the serum will hæmolyse red cells Large amounts of serum, which have been kept at 4 C will require a certain time before the whole volume reaches a temperature of 56 C and allowance should be made for this in addition to the 30 minutes required for proper inactivation In practice it has been found that even one hour at this temperature does not harm the anti globulin antibodies

The next step is to test the anti globulin serum for freedom from hæmolysins A mixture of 0 02 ml of the serum with 0 02 ml of a 10 per cent suspension of fresh human group O cells in saline is incubated at 37° C for 2 hours The cells will be agglutinated by the species specific antibody but they should not be hæmolysed If

hæmolysis does occur the serum must be further heated at 56° C

Once the serum has been freed from hæmolysins it may be put through the next stage of preparation, or it may be stored at −20° C until time permits of further processing

Treatment of the Clot

The bulk of the anti globulin antibody has been removed with the supernatant serum, but a surprising amount can be obtained from the residual clot by cutting it into small pieces (4-5 cm cubes) and by shaking these up in isotonic saline The mixture should be shaken for 5 10 minutes after which it is centrifuged and then the supernatant saline is removed and retained The process may be repeated at least once more These washings contain both anti globulin and a small amount of hetero antibodies which will have to be absorbed out before the reagent can be used The solution may store well if kept at −20° C and can be used as an anti globulin reagent by itself or it can be employed as a diluent for anti globulin sera

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CHAPTER V

THE PROCESSING OF ANTI HUMAN GLOBULIN SERUM

The Preliminary Examination

Previous chapters have described the immunisation of the animal, the collection of blood from it, the separation of serum from the blood and the inactivation of complement and destruction of hæmolysins present. Before carrying out the laborious process of removing the unwanted anti-species antibodies it is wise, at this stage, to examine the serum to see whether it is likely to be good enough to warrant further action.

The exact technical procedure is described in Chapter VI and the reader is advised to make himself familiar with the details and fallacies of the test before undertaking it. However, the principle of the preliminary examination is simple. We want to know what are the relative amounts of anti human species antibodies and anti-human globulin antibodies in the animal serum we have collected. Serial dilutions (from 1/1 to 1/1024) of the serum are made by adding saline. Then, using a *clean* opal glass tile, each dilution is tested against (1) a 50 per cent suspension in saline of thrice-washed human group O Rh (D) positive red cells, and (2) a saline suspension of the same washed cells which have been coated with incomplete anti Rh (D) antibodies. The results of testing two typical sera are shown in table I.

The serum of Rabbit A has agglutinated the untreated red cells at a dilution of 1/4 as shown in the uppermost line of results. This is a measure of the quantity of anti human species antibody present. The second line shows the dilution at which the same rabbit's serum will agglutinate red cells

TABLE I

	Dilutions of Rabbit Serum in Saline										
	1	2	4	8	16	32	64	128	256	512	1024
Serum from Rabbit A											
Unsensitised red cells	V	+	(+)	–	–	–	–	–	–	–	–
Coated red cells	V	V	V	V	V	V	++	++	+	(+)	–
Serum from Rabbit B											
Unsensitised red cells	V	++	+	+	(+)	–	–	–	–	–	–
Coated red cells	V	++	++	++	+	+	(+)	–	–	–	–

which have been coated with antibody and it indicates the presence of a considerable amount of anti human globulin in addition to the anti human species antibody For the serum to be worth processing the dilution at which it will react with coated cells should be at least twenty times the dilution at which it will clump unsensitised cells The serum from Rabbit A would therefore be regarded as satisfactory In contrast the serum from Rabbit B (the lower two lines of table 1), appears to contain little anti-globulin antibody and is unsuitable for further processing

Some laboratories on finding that an animal had produced a good titre of antibody would kill it and obtain the maximum yield of serum This practice is not only unnecessary but also unwise, since the " spectrum ' of anti globulin antibody will be less wide than that of an animal which has been further immunised

If, after the initial course of immunising injections, a rabbit has failed to develop a satisfactory titre of anti-globulin, it should be rested for 6 to 9 months before being given a second course If the animal again fails to respond sufficiently it should be destroyed or used for some other purpose The sera from animals which have responded inadequately, whether obtained from test samples or after death need not be discarded, they should be saved, pooled and inactivated After removal of the unwanted hetero-agglutinins the pool can be used as a diluent for good anti human globulin sera, thus diminishing the deterioration which tends to occur on storage, even at −20° C, of serum diluted with saline Most rabbit anti-human globulin sera can be diluted with an equal volume of such a " neutral " pooled serum and sometimes, with several volumes

The Removal of Unwanted Antibodies

It will be found, particularly when working with rabbit serum, that human red cells of the separate ABO groups

are not all agglutinated to the same titre by hetero antibody, since many rabbits have an apparently naturally occurring anti A-type agglutinin and an occasional one will have antibody which simulates the action of anti B

The hetero antibodies and anti human species agglutinins can be removed from anti globulin serum by absorption, by dilution or by heat treatment Whichever method is used the final product must not agglutinate fresh, well washed, group A_1 B and O human red cells

1 *Absorption Method*

For this procedure sterile fresh, well washed human red cells of group O and group A_1B are required If A_1B cells are not available half quantities of A_1 and B cells may be substituted

A suitable anti coagulant in which to collect the blood is 3 8 per cent of trisodium citrate ($2\ Na_3C_6H_5O_7 11H\ O$) in distilled water mixing one volume of anti-coagulant solution with four volumes of blood The solution is autoclaved and the blood when taken must be kept sterile The blood should be stored at 2 6° C and preferably the red cells used for absorption should be no more than four days old Older red cells more readily break down and are not so efficient in removing antibodies

When the blood has been withdrawn it is refrigerated until the red cells have sedimented The next step is to free these cells from all traces of plasma The plasma is aseptically removed replaced with sterile isotonic saline and the red cells and saline thoroughly mixed The mixture is then centrifuged to sediment the cells the supernatant is removed, replaced with more saline mixed and again centrifuged If possible the red cells should be kept sterile and a closed apparatus for the handling of large volumes of blood has been described[1] The cells will require at least six washings to free them from all plasma To determine

whether this has been achieved the sulpho salicylic acid test for proteins is useful Take 5 ml of the saline from the last washing and to this add 0 5 ml of 25 per cent sulpho salicylic acid If the washing has been efficient no trace of turbidity should be seen in the mixture

The washing process is time consuming but must be *thorough, since traces of plasma present may contain* sufficient globulin to neutralise the anti globulin antibodies in the animal serum during absorption Furthermore, the cells of each donor must be washed separately before they are mixed since reactions can occur between the plasma of one blood and the red cells of another which cause the agglutination of the red cells

At last we come to the actual absorption procedure which is performed in two stages The anti human globulin serum is, firstly, well mixed with an equal volume of the group O washed packed cells and is then stored overnight at 4° C during which time anti species antibody is adsorbed to the cells The O cells are then removed and discarded and a second absorption is carried out with A_1B (or A_1+B) cells After this absorption the anti-globulin serum is tested for reactions against A_1 B and O cells If hetero antibody has been incompletely removed further absorptions will be necessary, but they should be kept to a minimum since, as shown by Stratton and Jones (1955) [2] even well-washed cells will reduce the titre of anti-globulin antibody

2 *Dilution Method*

When an animal has developed in its serum an anti-human globulin antibody of high titre together with a comparatively small amount of anti human species antibody, it may be possible so to dilute the serum with saline that it no longer agglutinates well washed human A_1, B and O cells, but it still retains its capacity to clump red cells coated with incomplete antibody globulins The disadvantage of this

method is that, although the diluted serum may be a satisfactory reagent with which to detect the presence of those incomplete antibodies which are gamma globulins, it may, because of the dilution, dangerously fail to demonstrate the presence of incomplete antibodies which are non gamma globulins Since serum mixed with saline deteriorates on storage the dilution should not be made until required for use

3 *Heat Treatment*

In 1950 van Loghem[3] showed that hetero agglutinins could be destroyed by heating the animal serum to 63° C for 1-2 hours Subsequently other workers have varied the time and temperature required Menolasino and Davidsohn (1954)[4] found exposure of the serum to 70 C for 30 minutes to be effective, Hunter and Thomas (1957)[5] discovered that a temperature of 63° C for 30 minutes was sufficient for some sera However these authors and later Wilson, Simmons and Semple (1958)[6] have demonstrated that heat treatment may impair the serum s ability to react with some globulins To avoid coagulation of the protein by these temperatures, the serum should be mixed with an equal volume of saline before heating This method has certain advantages in a laboratory where the supply of human red cells for absorption is meagre or when the volume of serum is small or when the loss of anti globulin antibody during absorption would be too great

The Preparation of Anti Globulin Sera for Other Animal Species

Only the absorption of anti human globulin sera has so far been mentioned, but anti globulins for other species can be prepared by an analogous method For example if a rabbit anti goat globulin serum is required the inactivated serum from a rabbit which has been immunised with goat serum is absorbed with well washed goat red cells

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CHAPTER VI

STANDARDISATION OF ANTI HUMAN GLOBULIN SERUM

THE anti globulin serum which has been prepared will be used principally for the detection of incomplete antibodies of various titres and specificities Therefore, before this reagent is used it is necessary to know whether it will disclose the presence of a weak antibody, whether it is capable of demonstrating the existence of a range of different antibodies, and at what dilution it is best used

In blood transfusion laboratories the antibody most commonly encountered is the Rhesus antibody, anti D, and it is essential therefore in these laboratories, to determine the optimum dilution of the anti globulin which will agglutinate group O Rhesus positive red cells which have been sensitised by a weak incomplete anti D serum It must be remembered, however that what is an optimum dilution for the disclosure of Rhesus antibodies may be too dilute to demonstrate an antibody against another red cell antigen e g anti Kidd Furthermore the anti globulin serum may only be able to detect Rhesus antibodies and may be quite useless for the detection of other incomplete antibodies, i e the serum lacks anti globulins to these particular antibodies A more complete explanation of this is given in the next chapter

Ideally, the anti globulin serum should be standardised for each type of antibody which it will be required to detect In practice this is hard to achieve because of the difficulty of obtaining different types of antibodies for coating red cells A schedule of proven worth followed in the Sheffield

laboratory, is to test each dilution of the anti globulin serum against

1 Rhesus positive cells sensitised with a selected weak, incomplete anti D antibody
2 Rhesus positive cells of type CDe/cDE sensitised with weak, incomplete anti E antibody
3 Kell positive red cells sensitised with weak anti-Kell antibody (this is a selected "non gamma" type of antibody, see next chapter)
4 A_1 cells coated with incomplete anti-A antibody
5 If available, red cells from a case of the "cold antibody" type of acquired hæmolytic anæmia
6 If available, red cells sensitised with a weak anti-Duffy antibody

From the results obtained we hope to find two dilutions of the anti globulin serum at which all these antibodies have been detected, and it is then used at these dilutions only If we find the anti globulin is only reliable for the detection of Rhesus antibodies it is either pooled with another serum which will detect all types of antibodies, or it is only used in parallel with another anti globulin serum which will detect these antibodies

Anti globulin sera available may be supplied undiluted or they may have been diluted before issue If the raw serum is supplied it is customary to show on the label or on the accompanying report the dilution for the detection of Rhesus antibodies only The user of such serum may be unable to standardise the serum for the detection of other antibodies In this case the problem is overcome by employing serial dilutions of the anti globulin serum or, in a less certain manner, by using a dilution of the anti-globulin serum which is about one fifth of that recommended for the detection of Rhesus antibodies

If it is known that the serum issued is already diluted, or if the supplier states that it is suitable for use without

further dilution, then the user should be informed or should determine that the reagent is suitable for detecting all types of antibodies One of us (I D) has examined some commercially available anti globulin sera and found they have been so diluted that they will only detect Rhesus antibodies No indication of this was given in the literature issued with the reagent, and the usage of such materials can have dangerous consequences in blood transfusion work

Method of Standardisation

For this test coated cells are required which have been subjected to varying degrees of sensitisation Each portion of these cells is then mixed with a variety of dilutions of anti globulin serum and the results of the interaction between the cells and serum are noted

A *The preparation of the coated cells*

1 Select a weak, incomplete anti D serum which will agglutinate cells suspended in albumin at a titre of 1/16 to 1/32 but which is not active at greater dilutions
2 In tubes (9 mm by 75 mm) prepare serial dilutions (1/1 to 1/64) of this anti-D serum so that 1 ml of each dilution is available
3 To each dilution add 0 5 ml of a 50 per cent suspension in saline of washed packed, group O Rh positive cells
4 Incubate each mixture at 37 C for 30 minutes
5 Centrifuge each tube remove the supernatant and add at least 20 volumes of saline, flushing the cells up from the bottom to ensure thorough mixing
6 Centrifuge again at 800 1000 r p m to sediment the cells, remove the supernatant saline and replace with more saline until the cells have been washed at least three times
7 Prepare 50 per cent suspension in saline of these washed cells

8 Prepare also 50 per cent suspensions of thrice-washed A_1, B and O cells which have not been exposed to any antibody

B *The preparation of serial dilutions of the anti globulin serum*

Prepare serial dilutions of the anti-globulin serum in saline from 1/1 to 1/1024 or more, according to the titre which it is expected to reach, so that 0 5 ml of each dilution is available

C *The interaction between cells and serum*

1 A large white opal glass title measuring 20 inches square is well cleaned with soap and hot water to remove any traces of serum or globulin left by previous tests After drying with a *clean* cloth, kept specially for this purpose, the tile is marked out into the squares as shown in fig 7

2 Place one drop of the anti globulin serum in each square of the vertical columns so that every dilution can be tested against progressively less sensitised cells and against the non sensitised A_1, B and O cells In the last column drops of saline are used in place of anti globulin serum

3 Moving from left to right add one drop of sensitised cells (and then the unsensitised cells) to the squares containing each dilution of the anti globulin serum Carefully rinse the pipette in saline before taking up each new batch of cells

4 Mix each lot of cells and serum with the end of a clean plastic rod, rinsing and drying the rod between each mixing A quicker alternative is to use short lengths of wooden swab stick to stir the mixture and to discard each one as it is used It is advisable to have two people doing the mixing together in order to save time and to prevent the drops from drying too much before the results can be read

5 After 1-2 minutes (timed by the clock) rock the tile very gently back and forth and from side to side Let it stand for a further minute and then repeat the rocking

6 After it has stood for 5-7 minutes the results should be read Check the absence of agglutination with a hand lens in all apparently negative reactions

A H G Dilutions

		1	2	4	8	6	32	64	128	256	512	Cells and Saline
R.B Cs sensitised with anti D diluted	1											
	2											
	4											
	8											
	16											
	32											
	64											
Non sensitised cells	A_1											
	B											
	O											

FIG 7

THE INTERPRETATION OF RESULTS

When a rabbit has been immunised for the first time the results of the anti globulin serum standardisation test are likely to be of the pattern shown in table II The absence of reaction between the anti human globulin serum and the unsensitised A_1 B and O cells confirms that the anti species antibody has been satisfactorily absorbed No reaction should occur between the cells and saline, thus establishing that the anti D serum is truly incomplete Provided that these control tests are satisfactory examination of the remainder of the results will indicate to what degree the

TABLE II

A H G Dilutions

		1	2	4	8	16	32	64	128	256	512	Cells and Saline
R B C.s sensitised with anti D diluted	1	+++	+++	+++	+++	+++	++	+	(+)	(+)	–	–
	2	+++	+++	+++	+++	+++	+	(+)	(+)	gw	–	–
	4	++	++	++	++	+	+	(+)	(+)	–	–	–
	8	+	+	+	+	(+)	(+)	–	–	–	–	–
	16	(+)	(+)	(+)	(+)	gw	gw	–	–	–	–	–
	32	gw	gw	gw	gw	–	–	–	–	–	–	–
	64	–	–	–	–	–	–	–	–	–	–	–
Non sensitised cells	A_1	–	–	–	–	–	–	–	–	–	–	–
	B	–	–	–	–	–	–	–	–	–	–	–
	O	–	–	–	–	–	–	–	–	–	–	–

anti globulin serum can be diluted The results shown in *table* II *suggest that this serum would prove satisfactory at* a dilution of 1/8 to 1/16 for the detection of anti D antibodies

The Standardisation for Other Globulins

For this standardisation, each dilution of the anti globulin serum is tested against cells coated with the same amount of antibody The type of result obtained is given in table III

The Zoning Phenomenon

A second pattern of reactions is seen in tables IV and V This picture is obtained by using anti globulin sera from much immunised rabbits though it occurs less commonly in goat sera For this reason Hill and Haberman (1954)[1] suggest that ' further studies should be made to determine if the goat may not be superior to the rabbit with respect to the quality as well as the quantity of anti globulin sera produced '

The configuration of results in tables IV and V shows what is known as the *zoning phenomenon* Its occurrence in anti globulin sera was first described by van Loghem (1950)[2] and was later reported by other workers van Loghem, Kresner, Coombs and Fulton Roberts (1950)[3] and Hubinot (1951)[4] The lack of reaction where the antibody is used neat or in low dilution is known as the pre zone or prozone In the next few tubes where agglutination does occur is the reaction zone and subsequently we reach the point where no observable reaction has occurred the post zone

Certain findings suggest that the prozone phenomenon could be due to a relative excess of anti human globulin antibody over globulin on the surface of the red cells For example if additional sensitised red cells are added to a mixture of unagglutinated sensitised cells and anti human globulin serum in the prozone area agglutination takes place Also if we add a small amount of a very dilute

TABLE III

	Dilutions of A H G Batch No 1093											
	1	2	4	8	16	32	64	128	256	500	1000	2000
Cells sensitised with												
Anti Duffy (Grimes)	−	−	w	+	++	++	++	++	++	++	++	(+)
Anti Lewis (Le[a] 1556)	+	+	w	+	+	+	(+)	w	w	−	−	−
Incomplete immune anti A	++	++	+++	+++	+++	++++	++++	++++	++++	+++	++	+
Anti Kell (1370)	−	−	−	w	+	+	++	++	++	++	++	+
Cells not sensitised												
A_1	−	−	−	−	−	−	−	−	−	−	−	−
B	−	−	−	−	−	−	−	−	−	−	−	−
O	−	−	−	−	−	−	−	−	−	−	−	−

solution of gamma globulin to the unagglutinated sensitised red cell mixture, thereby lessening the amount of anti globulin present, clumping will occur The weaker the cell *suspension the more marked is the prozoning and this is* one of the reasons why, in performing the Direct anti globulin test, it is necessary to have a thick cell suspension at least (50 per cent cells in saline) to reduce the risk of false negative reactions

There may also be another explanation of the prozone It can be shown experimentally that the unagglutinated sensitised cells of the prozone area are, in fact, doubly coated with an inner layer of incomplete anti D and an outer covering of rabbit anti human globulin If these unagglutinated cells are washed three times with large volumes of saline and tested with goat anti rabbit globulin *serum they are agglutinated Since anti rabbit globulin* sera caused this, it shows that the cells are coated with rabbit globulin and this must be the rabbit anti human globulin, and this in turn must be attached to the Rhesus antibody which in turn is attached to the red cells This would suggest that the prozone may be due to incomplete antibodies in the rabbit anti globulin which coat the red cells and block the reactions of complete antibodies present in the anti globulin serum It can be further inferred that dilution of the anti globulin serum with saline dilutes the incomplete antibodies so that they no longer block the action of the complete antibody

It is obvious from studying the results in tables IV and V that either explanation applies only to the anti globulin antibodies detecting Rhesus antibodies, since the other antibodies are detected with less diluted anti globulin serum Therefore this would suggest that a zoning anti globulin serum may contain at least three types of antibodies—complete and incomplete anti globulin detecting Rhesus antibodies and complete anti globulins reacting with other

TABLE IV

		Dilutions of Anti Human Globulin Serum															Saline and R B C.s
		1	2	4	8	16	32	64	128	256	512	1024	2048	4096	8192	16384	
R B C.s sensitised with anti D diluted	1	−	−	(+)	+	+	++	++	++	+++	+++	++	+	(+)	(+)	−	−
	2	−	−	−	(+)	+	+	++	++	+++	+++	+++	+	(+)	−	−	−
	4	−	−	−	−	−	(+)	+	++	++	++	++	+	(+)	−	−	−
	8	−	−	−	−	−	−	(+)	+	+	+	+	+	(+)	−	−	−
	16	−	−	−	−	−	−	−	−	(+)	(+)	(+)	(+)	(+)	−	−	−
	32	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	64	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
non sensitised cells	A_1	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	B	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
	O	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

TABLE V

	Dilutions of Anti Human Globulin Serum											
	1	2	4	8	16	32	64	128	256	512	1024	2048
Washed sensitised R B C s	−	−	−	+	++	+++	++++	++++	+++	++	+	−
	Pre-zone or Prozone			Reaction Zone								Post zone

antibodies such as certain anti Kells In the next chapter further evidence will be given that anti human globulin serum contains more than one type of antibody

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CHAPTER VII

COMPLEXITIES OF THE ANTI GLOBULIN SERUM

So far human globulin has been frequently referred to as though it were a single entity and only a brief indication given that anti globulin sera have antibodies against different types of globulins By electrophoretic or chemical means alpha- beta- and gamma-globulin fractions can be distinguished in serum, and still further subdivisions of these fractions can be made Coombs and Mourant (1947)[1] established that Rhesus antibodies were mostly, if not entirely, in the gamma globulin fraction of the serum That being so, an anti human globulin serum, optimally diluted for the detection of anti-Rh antibodies, will also demonstrate the presence of other antibodies which are gamma globulins but it may fail to reveal the existence of antibodies which are non gamma globulin in nature

An illustrative example is shown in table VI An anti-human globulin serum was tested against Rhesus positive cells coated with incomplete anti-D antibody and the optimum dilution proved to be 1/128 When the same anti globulin serum was tested against blood from a patient suffering from acquired hæmolytic anæmia, whose cells were coated with ' cold " type antibody, the agglutination pattern was so different that, had the anti globulin serum been used only at a dilution of 1/128, it would not have demonstrated the fact that cells were coated with antibody

Dacie (1950),[2] Crawford and Mollison (1951)[3] and Renton (1952)[4] reported observations which showed that anti-globulin sera could be absorbed with one type of sensitised cell without removing the activity against other types of sensitised cells Using this technique, they demonstrated that anti globulin sera had at least two types of

TABLE VI

A H G Dilutions	1	2	4	8	16	32	64	128	256	512	1024	Cells and Saline
A H A cells	+++	+++	++	+	(+)	–	–	–	–	–	–	–
Rh coated cells	–	–	–	(+)	+	+	++	+++	++	+	–	–
Non sensitised cells	–	–	–	–	–	–	–	–	–	–	–	–

antibodies—anti gamma globulins reacting with Rhesus antibodies and some others, and anti non gamma globulins which failed to react with Rhesus antibodies but would detect other types The techniques which these authors describe can be used to prepare anti globulin sera that have either no anti gamma globulins or no anti non gamma globulins Renton (1952)[4] referred to the anti-gamma type as Fraction I and the anti non gamma as Fraction II The use of this terminology is confusing since preparations of human gamma globulin are often referred to as Fraction II and, therefore, the terms anti-gamma and anti non gamma are preferred The anti gamma globulin titres are invariably higher than the anti non gamma globulin titres

An anti globulin serum capable of detecting all types of globulins is sometimes referred to as a "wide spectrum reagent" In Chapter IV various methods of production of anti globulin sera using different antigens were described When injections of whole serum are used as antigen the animal is likely to develop both anti gamma and anti non-gamma globulins Even when gamma globulin is used as antigen the resultant animal serum may have anti non-gamma globulins, suggesting that the gamma globulin was not sufficiently pure Possibly in the future purified globulins may be used as antigens and specific anti-sera prepared

A different approach to making specific anti sera was used by Cutbush Crawford and Mollison (1955)[5] They studied the effect of the addition of dilute solutions of various globulin fractions (alpha, beta, gamma and $gamma_2$ globulins) to anti-globulin sera, and with these neutralised sera they tested cells coated with a variety of different incomplete antibodies They also obtained, from a patient suffering from a condition known as agammaglobulinæmia, a serum which contained both alpha and beta globulins, but no gamma globulins This serum when added to anti globulin sera gave them reagents lacking in certain types of

anti globulins and these were tested against red cells coated with various types of antibodies Their results confirmed that incomplete anti Rh antibodies, the "warm type" of incomplete antibody of acquired hæmolytic anæmias, some examples of anti Kell and some anti Duffy antibodies are gamma globulins, and some are probably in the $gamma_2$ globulin fraction of the serum The 'cold type' antibody of acquired hæmolytic anæmia, the cold incomplete antibody of normal serum, Dacie (1950) and some anti Lewis antibodies are contained in the alpha and beta globulin fractions Other anti Lewis and anti Duffy antibodies were found to be a mixture of gamma and non gamma types

The role of complement in the anti globulin test has been previously mentioned and several workers have shown that some substance, possibly complement, in fresh serum is necessary for the coating of non gamma globulins to be detected An interesting experiment is to expose Lewis-positive cells to an anti Lewis serum lacking in complement The cells are then washed three times and divided into two lots One portion is tested with an anti non gamma globulin serum and no reaction will be observed The other portion is incubated for 30 minutes at 37 C with a selected* fresh human serum after which it is further washed three times and tested with the same anti non gamma globulin serum when agglutination will be observed Dacie Crookston and Christensen (1957)[6] consider that the components of complement required are C 1, C 2 and C 4 but not C 3 These findings underline the fact that unless one is using fresh incomplete serum for sensitising the red cells for the Indirect anti globulin test a non gamma globulin antibody may escape notice If the sensitising serum has been stored for more than a couple of days it may be necessary to add to it a volume of fresh human serum to ensure a sufficiency of complement

* Preferably from a person whose red cells are Le(a−b−)

Enough has now been written to make clear to the serologist the importance of knowing and appreciating the capabilities and limitations of each anti globulin serum with which he has to work He should be aware of the anti-globulin serum which has been rid of its hetero antibodies by dilution, or by heat treatment, since such manipulations may result in a serum which will detect only coatings of gamma globulins

Three methods can be employed with anti-globulin sera to ensure detection of both gamma and non gamma globulins The first is to use serial dilutions of the anti globulin reagent, say from 1/1 to 1/512—*the serial dilution anti globulin test* This technique is tedious and involves a lot of unprofitable work but it is necessary sometimes

The second method is to use the anti globulin sera in two dilutions, one at the optimum dilution for the detection of gamma globulins and the other at the dilution for detecting non gamma globulins For this to be a reliable technique the results of the standardisation test must be known though in practice the dilution required to detect gamma globulins is usually found to be about four to six times that needed for the detection of non gamma globulins

The third method is one used by some commercial firms Two different anti globulin sera are selected, one of which must be capable of detecting the non gamma globulins, the other can be one containing anti gamma globulins only the sera from goats and rabbits can be mixed The optimum dilutions for detecting the two types of globulins are determined The serum containing the anti gamma globulin is then diluted to its point of optimum activity with saline or saline washings from the clots (obtained as described), or with a mixture of saline and neutral (i e free from hetero-antibodies) animal serum This anti gamma globulin serum can then be used as a diluent for the other anti non gamma globulin serum in such a proportion that it will give a

reagent which will detect both gamma and non gamma globulins at the same dilution The prepared reagent can be issued already diluted, or by adjusting the volumes used in its preparation a single dilution factor can be recommended which will give a satisfactory reagent The simple calculation necessary for preparing 100 c c of reagent requiring no further dilution is given for guidance

Serum A—Dilution at which gamma globulins are detected—1/100

Serum B—Dilution at which non gamma globulins are detected—1/10

$$\text{Amount of serum A required} = \frac{1}{100} \times \frac{100}{1} = 1 \text{ c c}$$

$$\text{Amount of serum B required} = \frac{1}{10} \times \frac{100}{1} = 10 \text{ c c}$$

$$\text{Amount of diluent required} = 100 - 11 = 89 \text{ c c}$$

Antibody Analysis by Gel Diffusion Techniques

Agar diffusion techniques (Ouchterlony 1949 [7] and Oudin, 1952 [8]) show great promise as a means of analysis of anti globulin sera Vaughan (1956) [9] has reported on the analysis of anti globulin sera by these techniques

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CHAPTER VIII

THE TECHNIQUE OF THE ANTI GLOBULIN TEST

THE details of the test to be described are those used in human blood group and transfusion serology and in the study of human disease The principles are equally applicable to investigations in other animal species and in bacterial *immunology*

✓To the uninitiated, the many published accounts of the use of the test for the detection of antibodies, the study of red cell antigens, the investigation of post transfusion reactions, the diagnosis of hæmolytic disease, or in forensic work, may suggest that a great variety of methods are used *Furthermore laboratory workers, speaking of the Indirect'* and Direct' tests increase the impression that they are different techniques, but this is not so

Basically, the anti globulin test is performed in two stages In stage one, cells are exposed to the action of incomplete antibody in such a way that the antibody becomes attached *to the corresponding antigen on the red cell surface, in* stage two, the anti globulin serum is employed to demonstrate these incomplete antibody molecules present on the cell surface The difference between the Indirect and Direct methods rests solely on the fact that, in the Indirect technique the first stage is performed in a test tube, whereas, *in the Direct technique the first stage has already occurred* in the body before the blood sample was collected

For the sake of simplicity the technique of the anti globulin test will be described in terms of the basic routine procedure adopted for the detection of incomplete anti D —the antibody most likely to be encountered in hospital practice The reasons for certain recommended actions and

the various modifications of reagents, which may be useful under different circumstances, will be outlined later

THE INDIRECT ANTI GLOBULIN TEST

1 Four drops of the serum suspected of containing incomplete anti D antibody are placed in a test tube
2 Two drops of a 50 per cent suspension in saline of known ' group O D positive red cells are pipetted into the same tube and are mixed with the serum
3 Control tubes are also prepared Control (i)—the positive control—contains a mixture of the same group O D positive cells with a serum known to contain incomplete anti D Control (ii)—the negative control—contains a mixture of the same cells with a serum known to contain no antibodies
4 The three tubes, one containing the suspect serum and the two containing the control mixtures, are incubated for 1½ hours at 37° C
5 Towards the end of this period remove a small amount of the sedimented cells to a slide and examine micro scopically for any agglutination which would signal the presence of complete antibody
6 Provided that no agglutination has taken place, remove the supernatant serum and saline from the remaining cells in all three tubes The next step of the Indirect test is the first stage of the Direct test

THE DIRECT ANTI GLOBULIN TEST

The red cells with incomplete antibody molecules adherent to their surfaces must be washed completely free from serum so that the only globulin remaining is that of the antibody If the Direct test only is being done positive and negative control cells must be prepared at this stage

1 To the sedimented cells add a large quantity of sterile saline, flushing up the button of cells and dispersing them throughout the saline
2 Centrifuge the cell saline mixture at 800 1,000 r p m for 1-2 minutes
3 Remove the supernatant saline by pipette or by suction from a water pump The more expert, after a bit of practice can empty the saline from the tube by a quick flick of the wrist leaving the button of cells *in situ* at the base of the tube
4 Repeat the washing twice, or preferably thrice, and after the final spinning remove every drop of supernatant saline
5 During the intervals of cell washing an opal glass tile is thoroughly cleaned dried and marked out in one inch squares with grease pencil as shown in fig 8

FIG 8

Positive Control	Negative Control	Test	Cell Suspension Control
1 A H G plus Sensitised cells	2 A H G plus Untreated cells	3 A H G plus Test cells	4 Test Cells plus Saline

6 Using clean apparatus and unsoiled saline dilute the anti globulin serum according to the instructions applicable to the particular batch in use
7 Place one drop of anti globulin serum in squares 1 2 and 3 In square 1 add one drop of cells which have been sensitised with known anti-D To square 2 add one drop of the cells which were mixed with inert serum Squares 3 and 4 each receive one drop of the cells under test and in square 4 a drop of saline is put in place of the anti globulin serum
8 Mix the contents of each square thoroughly spreading them over an area of about ¾ of an inch

9 As soon as the mixing is complete start a stop-watch After 1-2 minutes rock the tile *very gently* and replace on the bench Repeat the rocking, again very gently, after a further minute or two and then leave standing until ready for reading 5 7 minutes from the time of mixing

10 Read macroscopically with the opal tile held over a light All apparently negative results should be scrutinised with a (×10) hand lens All controls must be read first

Serological Points to be Noted

The Test Serum

At the beginning of the test the serum suspected to contain incomplete antibody must be freed from its own native cells If the serum is likely to contain antibodies in the non gamma fraction complement must be present and this can be affected by the addition of fresh AB serum to the serum undergoing test This point was emphasised on one occasion when a hospital technician, searching for compatible blood for a patient, discovered an antibody by the Indirect anti globulin technique This same test, repeated 24 hours later in the reference laboratory, did not disclose the antibody until fresh serum was obtained from the patient and was tested within 8 hours The antibody proved to be anti Le^a and, as shown by *Mollison and* Cutbush (1955)[1] could have caused a transfusion reaction

Cell Suspensions

The best results will be obtained with fresh unrefrigerated red cells Cells which have been stored for a time in the cold may have become coated by cold incomplete auto-antibody present in the donor's serum and when they give

a positive anti globulin reaction it may be wrongly assumed that the test serum contains antibodies

When selecting blood for transfusion, which has of necessity been stored at a temperature of 4 6 C, one cannot avoid the use of stored, refrigerated cells however, sodium citrate has an anti complementary effect which prevents coating of the cells by cold incomplete auto antibody This is one of the reasons for the use of citrate in the pilot tubes of some blood transfusion services On the other hand, this same anti complementary effect of citrate may mask the auto antibodies of acquired hæmolytic anæmia and Roth (1954)[2] recommends that blood samples from such patients should not be collected into anti coagulant

To avoid false reactions and to give more clear cut results, cells must be washed free from their own serum or plasma before admixture with the sensitising serum

An important modification of the test was the intro duction of the use of enzyme treated cells Unger (1951)[3] and Unger and Katz (1952)[4] applied the anti globulin test to cells which had, as a preliminary, been treated with trypsin or ficin and it was by the application of this same technique to trypsinised cells that Plaut Ikin, Mourant, Sanger and Race (1953)[5] were led to the discovery of the antibody anti Jk^b However as demonstrated by Mollison and Cutbush (1955)[1] some antibodies such as anti Le^a and anti-P may cause lysis of enzyme treated cells when the cell serum mixture is incubated

During the process of sensitisation it is recommended that the relative amounts should be two volumes of serum to one volume of cells The reason for advising these proportions is that we wish to obtain cells coated with as much incomplete antibody as possible If cell suspensions of a range of concentrations are mixed with an incomplete antibody serum it is found that the weaker the cell suspension the more likely is it that agglutination will occur at this

stage, the stronger the cell suspension the more likely is it that the antibody will be absorbed, but the cells will not be agglutinated. Table VII shows the results obtained when a complete anti D serum was titrated against varying strengths of suspension of the same D positive cells.

It is inevitable that some cells will be lost during the washing process and allowance must be made for this, so that at the final stage of the anti globulin test an ample quantity of coated cells is available. Because of the prozone phenomenon, described in Chapter VI, a falsely negative reaction is more likely to occur when a weak cell suspension is mixed with the anti-globulin serum and the presence of an antibody may be missed.

Normally, the mixing of washed cells with the serum which is expected to sensitise them is performed at room temperature. Difficulties may arise, however, when compatible blood is being sought for a patient suffering from hæmolytic anæmia of the cold antibody type. Under these circumstances, it will often be found advantageous to heat the donor cells and recipients serum separately to 40° C and to mix at this temperature before incubating them for a minimum of 30 minutes at 37° C in a water bath. Since the incubation time is comparatively short, a warm air incubator is not satisfactory. Wootton (1951)[6] showed that 30 minutes' incubation is not long enough to produce the optimum results and that incubation periods of up to a maximum of six hours were required to give the strongest reactions. If possible, therefore, when sensitising cells for the Indirect anti globulin test, the cell serum mixture should be left for at least two and preferably four hours.

The thorough washing of the sensitised cells is a most important step in this test. As can be seen in table VIII, even so small an amount as one part of human serum in 4000 parts of saline will neutralise an equal volume of diluted anti human globulin serum. Moreover, the anti globulin

serum reacts more readily with free globulin than with antibody globulin attached to the red cells, possibly because the bound globulin has already used some of its combining sites whereas the free globulin has all its combining sites available. Careful attention must also be paid to the provision of apparatus free from contaminating globulin. Test tubes, pipettes, teats, glass tiles or microscope slides, unless scrupulously cleaned, may result in hours of wasted labour. If the thumb or finger is used to occlude the end of the test-tube during mixing, be sure first that it has on it no trace of human serum.

TABLE VIII

Effects of traces of human serum on the anti globulin reaction

Dilutions of Human Serum in A H G	Rh+ Coated cells+ A H G
1 in 1 000	–
1 in 2 000	–
1 in 4 000	–
1 in 6 000	gw
1 in 12 000	+
Control (no serum)	++

The saline for washing red cells is normally used at room temperature (18 -20° C). When cells have been refrigerated and have become coated with auto agglutinin it is sometimes advisable to wash with warm saline at 37° C. A blood specimen, collected from an infant's umbilical cord, may have been contaminated with Wharton's Jelly, becoming gelatinous at room temperature and in this instance, too, washing with warm saline may be more efficacious.

When Coombs, Mourant and Race [7] first described their test in 1945, the final mixing of the anti globulin serum with the sensitised cells was performed in a test tube. They read the results both microscopically and macroscopically though they reported that "in practice the macroscopic reading has in every case proved sufficient". A year later, Coombs, Mourant and Race (1946) [8] compared the tube and tile

TABLE VII

Antigen Strength R B C Suspension	Antibody Dilution 1	2	4	8	16	32	64	128	256	512	Titration Score
2 per cent	V	++	++	+	+	(+)	gw	—	—	—	43
6 per cent	V	V	++	+	(+)	—	—	—	—	—	36
12 per cent	V	+	(+)	w	—	—	—	—	—	—	19
24 per cent	+	(+)	gw	—	—	—	—	—	—	—	10
36 per cent	+	gw	—	—	—	—	—	—	—	—	7
48 per cent	gw	—	—	—	—	—	—	—	—	—	2
60 per cent	—*	—	—	—	—	—	—	—	—	—	0
80 per cent	—*	—	—	—	—	—	—	—	—	—	0
100 per cent	—*	—	—	—	—	—	—	—	—	—	0

* These cells gave a positive Direct anti globulin reaction showing that the cells had absorbed the antibody

serum reacts more readily with free globulin than with antibody globulin attached to the red cells, possibly because the bound globulin has already used some of its combining sites whereas the free globulin has all its combining sites available Careful attention must also be paid to the provision of apparatus free from contaminating globulin Test tubes, pipettes, teats, glass tiles or microscope slides, unless scrupulously cleaned, may result in hours of wasted labour If the thumb or finger is used to occlude the end of the test tube during mixing, be sure first that it has on it no trace of human serum

TABLE VIII

Effects of traces of human serum on the anti globulin reaction

Dilutions of Human Serum in A H G	Rh+ Coated cells+ A H G
1 in 1 000	—
1 in 2 000	—
1 in 4 000	—
1 in 6 000	gw
1 in 12 000	+
Control (no serum)	++

The saline for washing red cells is normally used at room temperature (18 -20° C) When cells have been refrigerated and have become coated with auto agglutinin it is sometimes advisable to wash with warm saline at 37° C A blood specimen, collected from an infant's umbilical cord, may have been contaminated with Wharton's Jelly, becoming gelatinous at room temperature and in this instance, too, washing with warm saline may be more efficacious

When Coombs, Mourant and Race [7] first described their test in 1945, the final mixing of the anti globulin serum with the sensitised cells was performed in a test tube They read the results both microscopically and macroscopically though they reported that in practice the macroscopic reading has in every case proved sufficient' A year later, Coombs, Mourant and Race (1946) [8] compared the tube and tile

techniques and stated that "a parallel test done in a tube at 37° C and read microscopically gave the same result as a tile test in every case" Nowadays most authorities prefer to use the tile technique having found it to be a more sensitive method Furthermore, if the degree of sensitisation of the cells is slight, the agglutinates formed are rather loose and are liable to be broken up while being transferred by pipette from tube to microscopic slide Opal glass is selected for the tile because it has a smoother surface and is freer from small pits than any other type It can be easily scrubbed clean with a soft brush liquid soap and hot water rinsed free from soap and dried with a special "serum free" cloth just prior to use

The Anti Globulin Serum

When investigating a patient s serum which may contain incomplete antibody the anti globulin serum should be prepared in two dilutions (*a*) for the detection of gamma globulins, and (*b*) for the detection of non gamma globulins Normally, the correct dilution for each globulin should be known before the reagent is put into use, but, if it is not, a dilution of one fifth of that recommended for the detection of Rh antibodies will often be suitable These dilutions must be made up in clean tubes using a new pipette and clean teat on each occasion or alternatively the pipette and teat, which is used for anti globulin serum only, can be stored in a clean dust free box The saline with which the anti globulin serum is diluted should be clean and uncontaminated by pipette rinsings When a second dilution of anti globulin serum is employed the squares 1 2 and 3 of figure 8 must be duplicated on the tile When an anti globulin serum is capable of detecting both gamma and non gamma antibodies at the same dilution, both types of positive control cells should be placed on the tile in separate squares side by side

The Mixing of the Reagents

During the mixing process, when the anti globulin serum and the sensitised and control cells are being brought into intimate contact, the tile should be standing on a level bench. The implement used for stirring may be a wooden swab stick, in which case a separate stick is used for each mixture. Sometimes a plastic rod is used and it must then be quickly washed and dried between each mixing. Race and Sanger (1954)[9] use the four corners of a microscope slide—a different corner is applied to each square of mixture. Although this method is effective, false positives may result, especially if the glass microscope slide is rubbed hard on the opal glass tile. Its occurrence appears to be related to the type, age and composition of the glass.

Further mixing of the reagents takes place during the subsequent rocking of the tile. It cannot be too greatly stressed that the handling of the tile should be *very gentle*. Race and Sanger (1954)[9] draw attention to the fact that when working with the less avid antibodies "it is wise to be very gentle with the tile and to tilt it only at the time of reading". Gentle rotation of the tile, keeping the cells towards the centre of the area, is of the utmost help in detecting the weaker incomplete antibodies.

If the tile is left standing so long that evaporation takes place the results can be difficult to interpret and, particularly when the laboratory temperature is high, the tests should be examined for agglutination 5 7 minutes after the mixing. If, however, the tile is kept in a moist chamber during the reaction time and evaporation is minimised, the tests are read at 5, 9 and 12 minutes after the mixing.

CONTROLS

No reliance can be placed upon test results which have been inadequately controlled. It must be shown that (1) the anti globulin serum is capable of detecting both types

of globulin coatings, (ii) the anti globulin serum is not giving false agglutination, (iii) the agglutination of the test cells is due to the action of the anti globulin serum and not due to other causes

To demonstrate the gamma globulin coating two volumes of thrice washed group O D positive cells are mixed with four volumes of a known weak, incomplete anti D antibody and are incubated at 37 C for one hour Thereafter these cells are washed at the same time and in the same manner as the cells under test

To confirm the non gamma globulin coating one volume of a 50 per cent suspension of thrice washed group O red cells is mixed with ten volumes of fresh human serum and is then refrigerated at 4 C for 1 2 hours during which time the cells will pick up cold incomplete antibody These cells are also washed at the same time as the cells under test but the saline used should be warmed to 37° C —Crawford, Cutbush and Mollison (1953)[10] These cells are sometimes referred to as " cold sensitised cells "

The negative control is provided by the same group O cells which have been exposed to a serum known to be free from coating antibodies They are washed too, at the same time as the test cells For preference these negative control cells should not be obtained from refrigerated blood in which the serum may contain cold incomplete antibody However if the blood was drawn into anti coagulant the anti complementary action of citrate will have inhibited union of antigen and antibody

A further cell suspension control is required in the last stage of the anti globulin test The test cells, supposed to have been coated with incomplete antibody, are mixed, not with anti globulin serum but with a drop of saline Agglutination of this mixture indicates that some cause other than the globulin/anti globulin reaction is at work Some authorities control the results still further by adding

well-washed sensitised cells to all apparent negative reactions If agglutination occurs then it demonstrates that sufficient anti globulin serum was there to agglutinate the original cells if they had been coated

Apparatus

During the course of the test a great many tubes are constantly handled and moved to and from the centrifuge and racks, and it is obvious that some foolproof method of identifying each tube must be devised Grease pencil markings are unreliable adhesive labels are more satisfactory Murray (1957)[11] has overcome the necessity for moving tubes during cell washing by designing a centrifuge with a head which will accommodate metal racks holding test tubes

Recording of Results and Their Interpretation

In registering the results of tests it is inadvisable to record them merely as positive or negative, the degree of agglutination should be assessed and scored from '+ to '++++' and the symbol — ' should be used to signify absence of agglutination The results of control tests should be read first

The incomplete antibody used to sensitise cells for the positive control is specifically chosen for its weak action and the degree of agglutination in square 1 of the tile is therefore, unlikely to exceed '+' A complete absence of agglutination in this square suggests either that the cells have failed, for some reason to absorb antibody or that the anti globulin serum has lost its capacity to demonstrate the antibody Under these circumstances, negative results obtained in square 3 (the actual test) cannot be relied upon and the whole exercise must be repeated

The negative control in square 2 should show no

agglutination since the cells have not been in contact with an antibody bearing serum If agglutinates do form in this square they are probably caused by anti human species antibodies which have been insufficiently removed from the anti globulin serum The tests should then be repeated using a different anti globulin serum

The cell suspension control composed of supposedly sensitised cells mixed with saline, should remain unagglutinated If clumps of cells develop in square 4 a comparison should be made between it and the degree of agglutination of the test (square 3) A stronger reaction in square 3 than in square 4 suggests that the serum used for coating the cells in addition to the incomplete antibody present, contains complete antibody which the subsequent cell washing has failed to remove completely, with the result that the cells in saline agglutinate when rocked on a tile The enhancement of agglutination when these cells are mixed with anti-globulin serum has been called by Sturgeon (1954)[1] the *anti globulin augmentation test* A serum containing a mixture of complete and incomplete antibodies can, after inactivation of the complete antibody by heat, be re examined by the Indirect anti globulin technique (Dunsford and Bowley, 1955)[13]

When the test cells and the cell suspension control both show the same amount of agglutination the whole procedure should be repeated, using warm saline at 37° C for the washing of the cells When the Indirect technique is being applied the serum and cells should be separately warmed to 37 C before mixing

A word of warning must here be given about the interpretation of the test itself A negative or positive reaction means very little when considered on its own The result may be false even when all the controls are apparently working correctly In the next chapter a more detailed account will be given of the possible fallacies

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CHAPTER IX

THE CAUSES OF FALSE REACTIONS

ALTHOUGH the number of anti globulin tests performed each day *must be considerable and the amount of experience* gained over the past thirteen years should have led to the development of fool proof methods it remains largely an experimental technique which is fraught with difficulty Each result must be interpreted as only a part of a serological or antigen antibody reaction A test performed without controls, whatever the result, is useless and may be dangerously misleading, and, as previously mentioned a negative or positive result by itself means very little even when the controls are working correctly

CAUSES OF FALSE POSITIVE REACTIONS

1 *Anti human species antibodies in the anti globulin serum*

Inadequate absorption may have been the cause on some occasions when hetero antibody is found in the anti globulin serum, but some sera although initially satisfactory, seem to regain the unwanted antibody on storage—Cappell and MacFarlane (1946)[1] This finding suggests that although the bulk of hetero antibody can be absorbed on to human red cells and is discarded with them, further amounts of antibody are apparently neutralised in the serum On storage this neutralisation breaks down and the unwanted *antibody becomes active again*

Workers who use commercially produced anti globulin serum may find that the manufacturer recommends its use at such a dilution that the anti species antibodies are inactive, but if a less dilute solution is required the hetero antibodies become effective again and confuse the issue It is perhaps appropriate to interpolate here a warning

about recommended dilutions and expiry dates of sera The titres quoted by the suppliers of serum are those found by laboratory tests before the serum is issued and are for guidance only Some sera store less well than others and nobody can guarantee that a particular serum will be specific at a particular titre at any particular time after issue Similarly, although the label on the serum container may show that it has not yet passed the expiry date, this is no guide to its suitability

The routine use of a negative control with every anti-globulin test will detect false positive results due to anti-species antibodies and if such antibodies prove to be present they can be removed from the serum by further absorptions

2 *Cold complete agglutinins*

The test result will be invalidated when these antibodies manifest their presence by agglutination of the cell suspension control They have probably originated in the serum with which the cells were sensitised and, if the blood from which it was obtained has been refrigerated, separation of the serum while the blood is still at 4° C will ensure that the bulk of the agglutinin is discarded with the unwanted cells, leaving only a minimum free in the serum When the aim is to free cells from such an antibody, Dunsford, Cowen and Malone (1951),[2] and Holland (1952)[3] recommend that they should be warmed to 37° C for 15-30 minutes and then washed in warm saline in the preparation for the final stage of the anti globulin test Rosenfield, Vogel and Rosenthal (1951)[4] warm the cells to 37° C for only 1-3 minutes before adding the anti globulin serum and claim satisfactory results

3 *Cold incomplete antibodies*

Considerable difficulty may be encountered when using cells derived from clotted blood samples which have been refrigerated for the cells may have become coated with the cold incomplete antibody commonly occurring in the serum

of normal healthy donors This is particularly the case in blood transfusion work if the pilot tubes provided contain clotted blood or a suspension of the donor's cells in their native serum Such cells may prove very troublesome when, during a compatibility test, they are mixed with an anti globulin serum which is rich in anti non gamma globulin fraction Coating of cells is particularly liable to occur also in refrigerated clotted blood samples in which the proportion of cells to serum is small as, for instance, in blood drawn from a grossly anæmic patient No firm conclusion should be reached, therefore about the antibody content of a serum under investigation until it has been shown that the test cells without exposure to the suspect serum are not agglutinated by the anti globulin serum

In the view of Spalt and Kinsell (1953)[5] ' the interpretation of a positive Coombs' test is valid only on specimens which have not been refrigerated prior to separation of cells from serum ' The avoidance of refrigeration will reduce the number of false positive reactions but it is not always practicable When blood must be stored in the cold it is preferable to mix it with anti coagulant

The cold antibodies already considered are those active at refrigerator temperature but occasionally an apparently healthy person will be found who has in his blood a powerful incomplete antibody which is able to coat his own cells at a temperature just below that of the body In consequence cells obtained from clotted samples of his blood will always give a positive Direct anti globulin reaction To overcome this difficulty blood from such a person must be collected into 3 8 per cent sodium citrate which has been previously warmed to 37 C

4 *Bacterial contamination*

Any of the reagents used in the anti globulin test may become infected and curious results may be produced

Contaminated cells may give a false positive anti globulin reaction which can be detected by the occurrence of agglutination in the cell suspension control If the anti-globulin serum is infected it may become pan agglutinating with the result that it causes clumping of all cells with which it comes in contact, including those of the negative control The unknown serum, which is being investigated, may be contaminated and cause pan agglutination as evidenced by agglutination of the cell suspension control

The only way to overcome this difficulty is by prevention Blood specimens should be tested as quickly as possible after collection and if they have to be transported to a distance, cells should be suspended in *Panocell* mixture (Cahan, 1955)[6] which consists of

> Equal parts of Alsever s solution with a drop of penicillin and streptomycin to give a final concentration of about 30 units and 0 005 mg per millilitre respectively "
>
> Alsever's Solution
>
> 2 05 per cent Dextrose
> 0 42 per cent Sodium chloride
> 0 8 per cent Trisodium citrate
> 0 55 per cent Citric acid in aqua dist
> The final pH should be 6 1

Sera can be sent in the liquid state if sodium azide is added to make a concentration of 1/1000 or they may be dried first Anti globulin sera should be stored frozen at $-20°$ C while not in use in the laboratory

5 *Inadequate cell washing*

Coombs, Saison and Joysey (1955)[7] describe a condition in which anti globulin serum, although carefully freed from anti species agglutinins, may clump red cells which have not been completely rid of their surrounding plasma or serum The anti globulin serum reacts with the plasma protein,

of normal healthy donors This is particularly the case in blood transfusion work if the pilot tubes provided contain clotted blood or a suspension of the donor s cells in their native serum Such cells may prove very troublesome when, during a compatibility test, they are mixed with an anti globulin serum which is rich in anti non gamma globulin fraction Coating of cells is particularly liable to occur also in refrigerated clotted blood samples in which the proportion of cells to serum is small as for instance, in blood drawn from a grossly anæmic patient No firm conclusion should be reached, therefore, about the antibody content of a serum under investigation until it has been shown that the test cells without exposure to the suspect serum are not agglutinated by the anti globulin serum

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isotonic saline mixed gently with the tests will disperse conglomerates but will not scatter true agglutinates However, prevention is better than cure The tests should be read before they begin to dry or, alternatively, the tile may be placed in a moist chamber, or covered with the lid of a box to reduce evaporation

7 *The effects of silica*

Stratton and Renton (1955) [8] and Renton and Hancock (1957) [9] have shown that saline or sodium citrate solutions, sterilised in bottles made of certain types of glass, may contain colloidal silica which can cause spontaneous agglutination of well washed red cells The presence of serum inhibits this reaction, but when washed cells are mixed on a glass tile with a glass rod, with the bottom of a test tube, or with the corner of a microscope slide, and when the anti globulin serum has been so diluted that the inhibitory effect of serum is virtually negligible, conditions are fitted to encourage the development of red cell agglutination which may be attributed mistakenly to the anti globulin serum The occurrence of this type of agglutination will depend upon the composition of the glassware employed and upon the amount of grinding between one glass surface and another Cells which have been stored in the frozen state in glycerol and have been subsequently recovered seem to be more prone to react to colloidal silica than do normal cells

A change of glassware of a different composition might abolish this cause of false positive reactions and various other arrangements might be made For instance, citrate and saline solutions might be autoclaved in plastic bags or sterilised by Seitz filtration or, providing that the saline is to be used within 24 hours of the preparation of the distilled water used in its manufacture, the solution need not be sterilised Wooden swab sticks or plastic rods can be used

precipitating and enmeshing the red cells—a state which can be explained by the phenomenon of co agglutination described by Bordet and Gengou (1911)[7a] The cells of some species are more susceptible than others to co-agglutination and Coombs[7] and his colleagues first observed its occurrence while applying the anti globulin test in a study of pig iso antibodies The same sort of reaction was seen by Dunsford, Cowen and Malone (1951)[2] when using human serum and cells and the appearance was described as "agglomerates of red cells, cylindrical, oval or sausage shaped, hollow or solid, scattered in a negative field These resembled somewhat blood casts in urine, but were quite unlike red cell agglutinates

The importance of adequate and proper cell washing before the admixture of anti globulin serum cannot be too emphatically stressed Inefficient washing gives rise more commonly to false negative than false positive reactions—a problem which will be dealt with later

Another difficulty of the same sort is an appearance of graininess ' in Indirect anti globulin tests When a serum is being investigated to determine whether or not it contains antibody, the cells which it is to sensitise should be washed at least twice before being incubated with the serum Otherwise even if the cells are subsequently well washed before the addition of the anti globulin serum the final reaction will be weak, grainy and very difficult for the inexperienced worker to interpret

6 *The effects of ' drying*

Anti globulin tests performed upon an open tile at room temperature (18 20 C) begin to dry round the edges after about 7-10 minutes and the resultant conglomeration of cells simulates agglutination Provided that the volumes of reagents used in all the squares are equal the same appearance will be seen in the negative control square A drop of

case of Pasteur pipettes it may be possible to store these and their teats in a separate box or large glass test-tube for use only for anti globulin sera, but failing this a new pipette and teat should be used

Dirty saline which had been used for rinsing pipettes should never be used in the anti globulin test, it is better to open a fresh bottle

If, when resuspending the button of cells in saline during the washing process, the test-tube is up ended with a finger or thumb over its mouth, the presence of serum, or even of perspiration on the finger tip may be enough to ruin the test—Holland (1952) [1]

In order to ensure that these difficulties will be recognised when they occur, it is recommended that the control cells and test cells should be washed at the same time The use of control cells left over from a previous test might result in a failure to notice that something had gone wrong with the current reaction

As an additional control and to demonstrate that observed negative results in both test and control squares are in fact, truly negative, one can add one volume of well washed, sensitised cells to each mixture showing no agglutination If agglutination of these cells now occurs within 5-7 minutes it will confirm that the anti globulin serum is active and that the originally observed negative result was correct

2 *Incorrect dilution of the anti globulin serum*

Attention has already been drawn to the fact that differing concentrations of anti globulin serum are required for the demonstration of gamma and non gamma globulins A high dilution may react with gamma globulin antibodies, but fails to reveal the non gamma ones Conversely, a lesser dilution may coincide with a prozone area so that a gamma globulin antibody escapes detection The inclusion

to mix the cells with anti globulin serum on the opal glass tile and, so long as vigorous rubbing is avoided, the corner of a microscope slide makes an effective stirrer It has been observed, however that glass slides stored for a long time particularly if the surroundings are damp, appear to cause trouble more frequently than do new slides

8 *Other agglutinating substances in saline*

Hopps (1958)[10] has reported the occurrence of false positive results when a faulty ion exchange resin apparatus was used to obtain the pure water with which the saline solution was prepared Traces of some metals or chemical substances in the resultant saline led to the agglutination of all cells including the negative control and the cell suspension control

CAUSES OF FALSE NEGATIVE RESULTS

1 *The neutralisation of anti globulin by traces of serum*

Although this cause has already been mentioned, it is by far the commonest error committed by the inexperienced worker There are five possible sources of the unwanted globulin incompletely washed cells, dirty glassware dirty rubber bungs dirty saline or dirty fingers

Hill and Haberman (1954)[11] gave most valuable advice in recommending that until the worker has become familiar with the test cells should be given four washings Holland (1952),[1] and Madden (1956)[13] stressed the need for avoidance of high speed centrifuging which would throw down the cells before they could be properly washed by contact with the saline

In the average laboratory it is practically impossible to segregate test tubes slides tiles and other glassware for use for the anti globulin test only so a strict routine of cleaning must be developed and adhered to which will ensure that all apparatus used in blood grouping is dirt free In the

the anti globulin serum When weaker cell suspensions are used for the test there will be an excess of anti globulin antibody and insufficient antigen on the coated cells with which it can combine—one of the causes of the prozone phenomenon described earlier

6 *Over-centrifuging in the tube technique*

Although agglutination can occur immediately the anti globulin serum comes in contact with strongly coated cells in a tube, one is not always handling powerful reagents and Holland (1952)[1] has recommended that the mixture should be allowed to stand for at least fifteen minutes so that interaction may be possible before the tube is placed in the centrifuge If the spinning is begun too soon, or if high speeds are used, there will be insufficient contact between antigen and antibody for a reaction to take place and false negative results will be obtained

7 *Poor storage of reagents*

Loutit and Robb-Smith (1946)[17] found that "suitable sera preserve their potency when kept at —20° C but, when diluted and kept at higher temperatures, potency falls off, although a diluted, absorbed serum can be used for at least a week without loss of activity Pickles,[18] in the same year, reported that one of her sera diluted to 1/10 and stored at —20 C, had lost half its strength after five months She observed that weaker solutions deteriorate more rapidly and that sera stored at 4° C fairly quickly become inactive She recommends that only sufficient serum for about three months' work should be absorbed, that it should be stored at —20° C and that when dilutions are being arranged only enough reagent for one week's work at a time should be made up On the other hand, Aspinall (1955)[19] was able to store certain batches of diluted sera for months at —20° C without loss of titre

of correct controls with each test performed makes certain that such errors will be noticed

3 *Lack of complement*

Before non gamma globulin antibodies coating the cells can be demonstrated by the anti globulin test certain components of complement must be present Since anti-coagulants are anti complementary, patients' plasma should never be used for compatibility tests Furthermore, the donor cells taken from a pilot tube containing anti coagulant must be rid of plasma and citrate by thorough washing before exposure to the patient's serum with which they are to be matched The patient s serum must be fresh (stored for less than 48 hours), otherwise additional complement should be supplied in the form of fresh AB serum Blood cells from patients suffering from acquired hæmolytic disease frequently give a positive Direct anti globulin reaction but this will be abolished if heparin anti coagulant is added to the blood Roth (1954) [14]

4 *The use of aged red cells*

The best results will follow when fresh cells are used for the test Cells stored in saline suspension may hæmolyse after a comparatively short time and, even when lysis does not occur cells known to have been coated with antibody loose that antibody into the surrounding saline in a few hours, Barrie and Krieger (1954) [15] Even in clotted blood kept at 4 C for several days cells known to have been previously sensitised will sometimes give a negative Direct anti globulin test The practice of sending saline suspensions of cells for examination through the post is to be strongly condemned

5 *The use of thin cell suspensions*

Ellis (1952) [16] has shown that better results and fewer falsely negative reactions are encountered when a 50 per cent (or stronger) suspension of sensitised cells is added to

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On the whole, although some anti globulin sera can be satisfactorily stored at 4° C it is preferable to maintain them at a temperature of —20° C which will reduce the chance of bacterial growth Quick thawing performed at 37° C will minimise precipitation and the solution must then be well mixed, otherwise it will be found that the top of the container holds only saline while the active principle is mainly confined to the lower part If the anti globulin serum is to be stored at 4 C it is advisable to add 1/10 000 Methiolate or 1/1000 sodium azide as a preservative

Some laboratories issue anti globulin reagent diluted ready for use The choice of diluting fluid may be governed by the local availability of material but saline should be avoided whenever possible Anti globulin serum obtained from rabbits may be diluted with other rabbit serum from which the hetero antibodies have been absorbed, similarly goat anti globulin serum can be mixed with goat serum When describing the preparation of anti globulin serum mention was made (page 25) of a method of obtaining additional antibody by elution from the blood clot Although this eluate is largely saline the antibody content more than makes up for the deterioration likely to follow the use of saline as diluent Rosenfield Vogel and Rosenthal (1951)[4] report favourably on the use of a 2 4 per cent solution of bovine albumin (Cohn s fraction V) in saline as a diluting fluid

Whatever the material used for dilution, the reagent, if it is to be relied upon must still contain enough anti non-gamma globulin antibody to detect cell coating by antibodies of this type and the only proof that the anti globulin serum is active will be the evidence supplied by the behaviour of the control tests

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various ways such as suspension in albumin, exposure to enzymes or application of the Indirect anti globulin method

The efficiency of the four most commonly employed techniques is compared in table IX where it will be seen that the anti globulin method is the most reliable single test although no claim can be submitted that it will pick up every antibody

TABLE IX

Antibody	Saline	Albumin	Enzymes	Anti Globulin
Anti A anti B	+	±	±	±
Anti Rh	∓	∓	±	±
Anti Kell	∓	±	∓	+
Anti MNSs	±	±	−	±
Anti Duffy	∓	∓	∓	+
Anti Kidd	−	−	−	+

Symbols used + = Excellent ± = Usually good
− = No use ∓ = Not reliable

The performance of compatibility tests takes time and occasionally the need for blood for the patient will be so urgent that the necessary delay of two or more hours required for matching the blood will allow the patient's condition to worsen For this reason the practice of classifying patients as Dangerous " or Safer recipients—Tovey (1950)[1]—has been widely adopted

The Dangerous recipient is a person who may have developed a blood group antibody as a result of earlier stimulation by blood transfusion, by intramuscular injection of blood or by a pregnancy in which a blood group incompatibility between mother and child existed The Safer recipient has no obstetric history suggesting maternal-fœtal incompatibility and has never received blood Before this classification can be relied upon, careful questioning and accurate answers are most essential Where any doubts exist, or if the patient, being unconscious cannot give the necessary information he should be regarded as potentially ' dangerous

CHAPTER X

APPLICATIONS OF THE ANTI GLOBULIN TEST

THE anti globulin test has its main usefulness in the preparation of blood for transfusion, in the study of hæmolytic disease of the newborn and in hæmolytic anæmia in adults Other more specialised fields in which it may be found helpful will be briefly described though the reader should consult the references for more detailed information before applying the technique to his particular problem

BLOOD TRANSFUSION

Before discussing the advantages of the anti globulin test as employed in blood transfusion work it may be profitable to review quickly some general principles

Transfusion of compatible blood can be a life saving procedure in replacing lost red cells and in restoring a depleted blood volume Before the transfusion is undertaken, however a compatibility test must be done to ensure that the donor's red cells will not be prematurely destroyed by an antibody in the patient's circulation, such cell destruction would do the recipient no good and might endanger his life

In the compatibility test (also known as the direct match or major cross match) cells from the donor are mixed with the patient s serum In spite of a great many hours of labour in many laboratories no single test has yet been invented which will unfailingly detect all types and strengths of antibody which may be present Donor's cells in saline suspension, when mixed with the patient s serum will disclose the presence of any complete antibody, while for the demonstration of incomplete antibody several tests have been devised which involve treatment of the donor s cells in

have disastrous consequences Resultant agglutination would show that an incompatibility between donor and patient existed and that it would be unsafe to give the transfusion until compatible blood from another donor had been found If no agglutination is shown by this technique it should not be assumed automatically that the bloods of the donor and patient are compatible, unless the saline and/or albumin compatibility tests also produce no agglutination

Clinicians confronted with the plight of a patient sorely in need of transfusion may, quite understandably, become peevish at the delay occasioned by compatibility tests and may urge that the time allowed for the various procedures should be reduced While sympathising with their frustration it must also be remembered that rapid techniques are less reliable and the accuracy of interpretation of their results depends to some extent upon the experience of the technician It is only when the risk to the patient of withholding blood outweighs the risk of giving possibly incompatible blood that departure from safer routine methods is justifiable

2 *The Rapid anti globulin compatibility test*

When the time available is not more than 30 minutes it may be permissible to shorten the period of incubation during which attachment of antibody to the red cell antigens takes place

Donor blood of the same ABO and Rh group as the patient is obtained Two volumes of a 50 per cent saline suspension of well-washed donor's cells is mixed with four volumes of the recipient's serum in a test tube The mixture is incubated for at least 15 minutes at 37° C in a water bath, which will more quickly bring the tube to the correct temperature than could be achieved in an incubator In order to bring the antibody and antigen into close association

The point of making this classification is that the " safer ' recipient is unlikely to have developed an antibody which will destroy the donor cells In his case therefore the cross match tests can be done by the comparatively straight forward and less time consuming saline and albumin techniques Infrequently, an antibody will be unexpectedly found in the blood of a patient whose history has not led one to suspect its presence In the majority of such cases it will prove to be an incomplete anti Rh antibody The albumin technique which is excellent for the detection of most incomplete Rh antibodies should therefore be included in the compatibility tests even when the patient belongs to the safer " category

Compatibility tests for a dangerous " recipient must involve more searching techniques Saline and albumin matching tests will disclose the majority of more commonly occurring antibodies but some incomplete antibodies are only detectable by the anti globulin technique and this is used in addition to the saline and albumin methods

1 *The anti globulin compatibility test*

The Indirect anti globulin technique is used Washed donor s cells are mixed with a double volume of the patient s serum and incubated for 1½ hours so that if an antibody against antigens on the donor s cells is present it will have an opportunity to become affixed to the red cell surface Complement may be necessary for eventual detection and this point should be borne in mind if samples of patients sera are stored in the refrigerator for several days before compatibility tests are to be done After incubation the cells are thoroughly washed free from unbound globulin In the final stage of the test the anti globulin serum should be used in at least two dilutions to make certain that both gamma and non gamma globulin antibodies are detectable Failure to use ' wide spectrum anti globulin serum can

especially where the volume of blood transfused has been relatively large the repeated saline and albumin compatibility tests may appear to give perfectly satisfactory results, however, by performing a Direct anti-globulin test with serial dilutions of anti globulin serum on the unrefrigerated cells obtained from the patient's post transfusion blood it may be possible to show that the cells are coated with antibody and that the transfused blood was probably incompatible

THE DETECTION OF BLOOD GROUPS ANTIGENS AND ANTIBODIES

The use of the anti globulin technique to investigate incompatibilities found during the selection or following the giving of blood has greatly advanced our knowledge of human blood groups Anti Duffy (anti Fy^a) was discovered by this method and some red cell antigens, such as E^u, can be detected apparently only by the anti globulin technique

Possibly the most important red cell antigen which is detected by the anti globulin test in blood transfusion work, is the variant of the Rhesus D factor—D^u It is characteristic of cells bearing the antigen D^u that they will be agglutinated by some anti D sera, but not by others A ' high grade D^u " blood contains cells which are agglutinable in saline suspension by some complete anti D sera, ' low grade D^u " cells are not clumped by any saline anti D but are sensitised by some incomplete anti-D sera Since it has been proved that the D^u antigen can immunise D negative patients causing the development of anti D antibodies, it is therefore essential to be able accurately to distinguish donors whose blood is really Rh negative (cde/cde) from those who appear to be Rh negative, but who have the antigen D^u (cD^ue/cde)

Since the saline and albumin agglutination tests are unreliable it is a routine practice in transfusion services

the tube is then spun in a centrifuge at 1000 r p m for 1 2 minutes After spinning remove a drop of cells for micro scopic examination Agglutination at this stage would demonstrate the presence of a complete antibody, the action of which might not be detectable if observations were not made until the cells had undergone their next stage of triple washing If no agglutination develops after the addition of anti globulin serum, the blood of the donor can be transfused with reasonable confidence

Whenever it has been necessary to curtail the technique in this way it is wise to confirm the results of the rapid test by immediately setting up saline albumin and anti globulin compatibility tests, allowing the full time for reactions to take place By the time these results can be read the blood will probably have been transfused but if by any chance the rapid method has failed to disclose the presence of an antibody, its subsequent discovery may enable the clinician to anticipate and treat any complications which may arise before irreversible changes have taken place in the patient s tissues

The Investigation of Transfusion Reactions

It happens, unfortunately from time to time that, as a result of a technical clerical or administrative error, a patient will receive incompatible blood When such a mistake has occurred or is suspected a sample of the patient's blood is collected for investigation forthwith, or at least within 24 hours of the development of the transfusion reaction The grouping and compatibility tests are repeated In spite of clinical evidence that a reaction has occurred it may be found that no free antibody is demonstrable in the patient's post transfusion serum This happens when the transfused donor cells mop up and become coated by the antagonistic antibody before being broken down in the patient's circulation Under these circumstances and

especially where the volume of blood transfused has been relatively large the repeated saline and albumin compatibility tests may appear to give perfectly satisfactory results, however, by performing a Direct anti globulin test with serial dilutions of anti globulin serum on the unrefrigerated cells obtained from the patient's post transfusion blood it may be possible to show that the cells are coated with antibody and that the transfused blood was probably incompatible

The Detection of Blood Groups Antigens and Antibodies

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throughout the world to use the anti globulin technique to examine donor bloods which are apparently D negative, and this is of particular importance in peoples of negro extraction in whom the genotype cD^ue/cde occurs more commonly than is found in white populations.

The most reliable and sensitive method is to expose the alleged D negative cells to the action of a pool of powerful incomplete anti D sera and then to examine the cells for a coating of antibody. Those cells which are unagglutinated by the anti D sera, but which absorb the anti D antibody and are subsequently agglutinated by the anti globulin test, are presumed to have the D^u antigen. The cells declared as D^u should give a negative Direct anti globulin test before exposure to the incomplete anti-D sera.

The Indirect anti globulin test has a further application in the investigation of sera containing immune anti A and anti B antibodies. Ideally, a patient should receive only blood of his own group but sometimes group O blood is transfused to patients of other groups in the mistaken belief that it is universally compatible. While in the majority of cases no harm results, it will be found, when dealing with large numbers of group O donors, that about 10 per cent of them have in their sera, alpha and/or beta hæmolysins and incomplete forms in addition to the naturally occurring complete or saline anti A and anti B agglutinins. The transfusion of O blood containing immune anti A to an A patient for instance might result in a harmful reaction.

One method employed to discover whether immune forms of anti A or anti B are present, in addition to the normal agglutinins in a group O serum is to neutralise the complete antibodies by the addition to the serum under investigation of A and B blood group specific substances (obtained in pure form or from the saliva of a 'secretor'). Immune forms of anti A and anti B cannot be neutralised in this way, but will be capable of coating A_1 and B cells as

evidenced by the Indirect anti globulin technique The donors whose sera are able to coat A_1 and/or B cells are classified as "Dangerous group O donors" and their blood should be used only for group O patients Some transfusion centres screen their group O donors and give a distinctive label to bloods which are considered unsafe for transfusion to patients of other ABO groups

Hæmolytic Disease of the Newborn in Man

At the time of conception both parents contribute genes to the new individual It happens not infrequently that the infant inherits from its father blood group antigens which the mother does not possess and against which it is theoretically possible for her to make antibodies So long as no antibodies are present in her circulation the blood group incompatibility between mother and child is of no consequence and can be ignored If she should develop antibodies, however, which can pass the placental barrier and disrupt the fœtal red cells, the child's life may be endangered

The mechanism of the stimulus to antibody production provided by transfusion where "foreign" red cells are introduced directly into the patient's blood stream can be fairly readily understood It is less easy to postulate a mechanism which explains how antibodies can be provoked by pregnancy alone, since the maternal and fœtal circulations are entirely separate and have no blood vessels in common by which fœtal red cells could gain access to the mother's blood stream

The most credible hypothesis illustrated in fig 9, suggests that sometimes during pregnancy a breakdown of adjacent maternal and fœtal blood vessels occurs and permits a small quantity of the child s blood to enter the mother s capillaries The break quickly heals but not before a number of fœtal cells sufficient to initiate antibody formation, have mingled with the mother's blood Cases have been observed

and recorded—Chown (1954 and 1955) [3] and Dunsford (1957) [4]—in which recognisable fœtal red cells have been found in the maternal circulation during pregnancy It may be that small accidental hæmorrhages of this sort can occur several times during the gestation period, each occasion

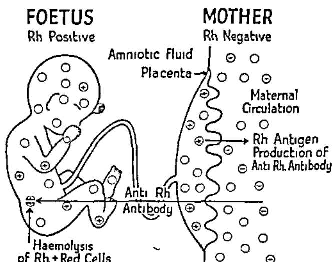


FIG 9 The mother is Rh negative The fœtus is Rh positive having inherited this character from its father Rh antigen (either as fœtal red cells or in soluble form via the amniotic fluid) passes from the fœtus to the mother who responds by producing anti Rh antibody The antibody passes from mother to the fœtus whose Rh positive red cells it damages leading to hæmolysis and so to anæmia and jaundice This process only takes place in a small minority of cases where an Rh negative mother has an Rh positive baby

giving additional impetus to the development of antibody, but it is more likely that the intermixing of bloods takes place at or about the time of parturition The antibody is then first detected in the post natal period and therefore will not affect the child whose red cells caused the immunisation of the mother, although a subsequent child possessing the same red cell antigen will be susceptible to the effects of the antibody

The immune antibody produced by the mother, being soluble and of sufficiently small molecular size, can diffuse across the placental barrier to attach itself to the fœtal red cells which are then destroyed or weakened, with the result that at birth the child may be found to be anæmic and quickly becoming jaundiced after birth In severe cases the breakdown of the cells is so great that the fœtus dies *in utero* or within a few days of delivery

Hæmolytic disease of the newborn occurs comparatively infrequently the incidence being only about 1 150 to 1 200 pregnancies Even where the blood-groups of the parents are glaringly incompatible immunisation of the wife and subsequent disease of the children is by no means inevitable It is fortunate too that hæmolytic disease is one of the few conditions affecting the newborn child which can be anticipated, making it possible to plan the treatment which may be necessary within a few hours after its birth

The causative antibodies most commonly encountered are those directed against the Rhesus blood group antigens The great majority of cases, probably 98 per cent or more, are due to the action of anti D antibodies, although occasionally anti C, anti E, anti c, anti f or anti C^w have been implicated Antibodies against other blood-group systems such as immune anti A, anti-K or anti k, may be found also to have been responsible for destruction of fœtal red cells

In order to be able to select and forecast those pregnancies in which hæmolytic disease is a potential hazard the mother's blood is usually examined once or twice during the course of the pregnancy to discover whether or not she is developing antibodies As a rule the use of ordinary saline and albumin agglutination methods, in which her serum is mixed with a panel of red cells of known genotype, will disclose any antibody present Some anti-Rh and anti Kell antibodies, however, are demonstrable only by the *Indirect anti globulin*

technique and this method should be included routinely, particularly when an anti natal patient presents who has a history suggesting the possibility that she has become immunised

If antibodies are found in the serum of a pregnant woman it is usual to recommend that her confinement should take place in a maternity unit with facilities for early clinical and serological assessment of the child s condition and where, if necessary within a few hours of its birth, the child may be treated by exchange transfusion of red cells which will not be destroyed by the maternal antibody freely circulating in the infant's blood stream

When the child of a mother who is known to have antibodies is observed immediately after birth to be obviously anæmic or jaundiced there can be little doubt about the probable clinical diagnosis of hæmolytic disease of the newborn However, when such a mother produces a child with no clinical signs of the disease it may mean either that the child *is affected* but the development of signs of hæmolysis is less well advanced than in the previous case or that the child is *unaffected* since its blood does not contain the antigen against which the maternal antibody is directed The performance of a *Direct anti-globulin test* on red cells obtained from the child s umbilical cord will distinguish those infants whose cells are coated by maternal antibody, and who are therefore suffering from hæmolytic disease, from those with uncoated cells who are unaffected

The circumstances in which it is especially important to collect and test red cells obtained from the umbilical cord are

1 When a multiparous D negative mother has had no examination of her blood during pregnancy,
2 In the second or subsequent pregnancies of a D negative mother Although examination of her blood in the 32nd to 35th week of pregnancy may have shown no abnormality

she may, occasionally, develop enough antibody in the later weeks of pregnancy to coat her child's red cells,

3 When ante natal tests have shown that the mother has become immunised to one of the red cell antigens,

4 When a D positive mother has an obstetric or transfusion history which would put her in the " dangerous recipient " category,

5 When the child has an unexplained anæmia or develops jaundice within three days of birth.

A knowledge of the reaction obtained in the Direct anti globulin test may be very helpful when a decision about treatment has to be made, but the diagnosis must not be allowed to hinge upon the results of this test alone. A negative reaction does not entirely exclude hæmolytic disease, nor does a positive result by itself prove the condition to be present. Certain other criteria must be fulfilled before reliance can be placed upon the results. It should be proved (i) that the mother's serum contains antibody active against the red cells both of the child and of its father, (ii) that the mother's red cells lack an antigen possessed by both child and father, and (iii) that the child's red cells are, or can be, coated by maternal antibody so that they give a positive Direct anti globulin reaction. (There may be exceptions to this rule when hæmolytic disease is due to ABO incompatibility.)

Fallacies of the Direct Anti Globulin Test

False Negative Results

1 If collection of blood from the baby is delayed for several days after birth there may be no coated cells left in circulation with which the anti globulin serum can react. Examination by saline, albumin and the Indirect anti globulin methods, of the ability of the mother's serum to agglutinate or coat her child's red

cells should make clear whether or not the child is affected by hæmolytic disease

2 When hæmolytic disease is due to ABO incompatibility the condition may be missed if the result of the Direct anti globulin reaction forms the sole diagnostic criterion Although Rosenfield (1955)[5] claimed that, using a modified technique, positive results were not infrequently observed, our experience and that of Mollison (1956)[6] is that the result is quite often *negative and that when positive the reactions are* comparatively weak When trying to establish the diagnosis the more reliable signs are (i) the presence of anti A or anti-B hæmolysins at 37° C in the mother s serum, (ii) the finding of incomplete anti A or anti B in the child s blood, and (iii) spherocytosis and an increased osmotic fragility of the child s cells (Dunsford and Bowley 1955[56], and Mollison, 1956)[6] A positive Direct test may sometimes be obtained by incubating the child s cells with fresh adult serum

3 The child may have had a transfusion before the blood samples were taken for testing (Jakobowicz, Krieger and Simmons 1948)[7]

False Positive Results

1 Contamination by Wharton's Jelly of blood samples collected from the cut end of the umbilical cord may cause the red cells to conglomerate, giving an appear ance of agglutination A similar picture in the cell suspension control should draw attention to the false nature of the result The blood sample should be warmed to 37 C the cells are transferred to saline at 37° C and are then rewashed before repetition of the test Contamination by Wharton s Jelly can be avoided by using a needle and syringe to collect the blood from the umbilical vein

2 A falsely positive Direct anti globulin test may result from the action of a cold incomplete antibody which had its origin in the mother's serum (Hollander, 1952)[8] This antibody is inactive at body temperatures and when it passes through the placenta to the infant's circulation it does no harm It is not until a blood sample, taken from the child, is allowed to cool that the free antibody present actively coats the red cells which then show a positive Direct anti globulin reaction If blood from the mother is stored in the refrigerator her cells, too, will give a positive result though the reactions of the cells of a group-A_1 mother may be weaker than those given by the cells of her group-O child The antibody in the maternal serum will be found to have no activity against fresh, unrefrigerated samples of cells from the child s blood from the father's blood or from the genotyped panel of donor cells

These deceptive results are not the only possible causes of difficulty, when anomalies are found consideration should also be given to the causes of false reactions previously mentioned on pages 35 64

Hæmolytic disease of the newborn cannot with certainty be diagnosed from an investigation of the baby s blood alone It is essential to examine the mother's blood as well and ideally the father's blood also should be tested On rare occasions the mother s blood does not appear to contain an antibody against a panel of cells and yet she gives birth to a baby whose cells give a positive Direct reaction An examination of the mother s serum for antibodies against the father s cells will help to determine whether the baby s positive Direct test was a false or true result

Any assessment of the severity of a particular case of hæmolytic disease is usually based mainly on clinical signs and on the hæmotological findings Wiener and Gordon

(1953)[9] considered however, that a *quantitative anti globulin test* can indicate the degree of affliction The baby s cells are washed thoroughly and are then tested against serial dilutions of a suitable standard anti globulin serum At the same time the anti globulin serum is titrated against D positive cells which have been sensitised by exposure to a standard anti D serum The ratio of the two titres multiplied by 100 represents the degree of coating of the child's cells Greenwalt and Wagner (1955)[10] applied this quantitative anti globulin technique in 86 cases and found no regular correlation between the test results and the severity of the disease

The After Effects of Hæmolytic Disease of the Newborn

Children surviving hæmolytic disease of the newborn without treatment and a small percentage of those who have been treated may have some degree of brain damage (Fulton Roberts, 1957)[11] As a result the child may present, months later, with athetoid movements of the limbs spasticity mental retardation unexplained deafness or hepatomegaly and splenomegaly (Drummond and Watkins, 1946)[1] —and the nature of the underlying cause may not be revealed by clinical examination The performance of an Indirect anti globulin test, using the mother's serum and the child s red cells, may give a pointer to the ætiology

Some women, known to have become immunised during pregnancy have no demonstrable antibody when the blood is examined a few months after the birth of an affected child, but most immunised women continue to produce antibodies throughout life In general the younger the child the greater the chance of demonstrating any blood group incompatibility existing between it and the mother How ever, it has been possible by the Indirect anti globulin test,

to demonstrate in the blood of a 70-year-old woman, immune antibodies against the red cells of her 40-year-old son

HÆMOLYTIC ANÆMIA

The name "hæmolytic anæmia" covers a number of conditions in which the main feature is a shortening of the life span of the red cells manufactured by the patient. Sometimes the underlying cause can be readily recognised, sometimes its origin and the reason for the hæmolytic crises which recur periodically are frankly unknown The field is too vast to attempt more than a brief description here The reader who wishes to study the fascinating confusion of the subject should consult Dacie's book, *The Hæmolytic Anæmias*, where he will find many of its complexities unravelled

Two broad categories can be distinguished In the *congenital* or *hereditary* type of hæmolytic anæmia an intrinsic defect in the structure of the red cell, or of its enzymes, apparently causes it to age more quickly than a normal cell Hereditary spherocytosis, sickle cell disease and Mediterranean anæmia are examples of this type of condition The label, *acquired hæmolytic anæmia*, is attached to a more diverse series of abnormal states As implied by the title the abnormality is not inherited, it is probable that the fault is in the environment of the cell rather than in the cell itself, although the extrinsic factor may so alter the cell structure that it becomes more vulnerable to normal destructive forces While it may be possible occasionally to attribute a hæmolytic episode to a bacterial infection or a chemical poison it more often happens that no such connection can be traced In some cases an auto antibody, which may be either an agglutinin or a hæmolysin, can be demonstrated by serological methods in the patient s blood, though the purpose or cause of such a self destructive mechanism is hard to understand

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stored in the refrigerator the red cells may become coated with the cold incomplete antibody commonly found in the sera of healthy people, so that they give a positive Direct anti globulin reaction which is not indicative of hæmolytic anæmia To avoid confusion of this sort, blood samples from patients suspected to be suffering from hæmolytic anæmia should be tested before they are allowed to cool To do this effectively the venepuncture should be made with a warm syringe, the blood should be collected into a warmed container and it should be kept at 37° C until the clot has formed The subsequent centrifuging and separation of serum should be carried out without delay Dacie (1954)[14] advocates that "erythrocytes sensitised by cold antibodies must be washed in saline warmed to 37° C in order to elute agglutinins' However, Dunsford and Bowley, 1955[56] have not found this necessary if the blood is collected as described above, since the cold antibody is given no chance to become bound to the cells Under these circumstances the use of saline at room temperature (18 20° C) for cell washing has proved satisfactory

To make it as certain as possible that the antibody coating the patient's cells will if present, be detected a *Serial dilution anti-globulin test* should be performed The technique is similar to that of the Direct anti globulin test except that the anti globulin serum should be prepared in doubling dilutions of from 1/1 to 1/1000 or more, depending upon the titres which were found to be optimum for the demonstration of gamma globulins If possible at least two different batches of anti globulin serum, both containing anti gamma and anti non gamma components should be employed Red cells coated with gamma and non gamma-globulin antibodies must be used as a positive control to test each dilution of the anti globulin serum, The tile technique is deemed to be the more sensitive and, indeed, Wright, Dodd, Bouroncle, Doan and Zollinger (1951),[15] and

Shortly after the description of the anti globulin technique was published Boorman, Dodd and Loutit (1946)[13] applied the test to a series of cases of hæmolytic anæmia and found that cells from patients suffering from the aquired form gave a positive Direct anti globulin reaction whereas cells from patients with the congenital type of disease did not It was thought at first that here was a splendid tool by which cases of acquired hæmolytic anæmia could be distinguished from other clinically similar conditions

Later experience has shown that a diagnosis cannot be made in so simple a manner and that the results of the anti globulin test should be interpreted with caution It is still true to say that auto antibodies are never found in congenital hæmolytic anæmia, they may be demonstrated in the majority of cases of the acquired form of the disease, but there are occasions when no antibody can be detected, perhaps because our armament of tests is still inadequate to reveal its presence This should not be taken to imply that the anti globulin test is of little value in hæmolytic anæmia On the contrary provided that it is appreciated that a negative test result does not necessarily exclude the disease, and that positive reactions can occasionally be found in other disease conditions and even in normal individuals, the information obtained from the anti globulin test can be most helpful

When an active auto antibody is found in the serum of a patient suffering from hæmolytic anæmia it may have the characteristic behaviour of either a cold or a warm antibody and may be either a gamma or non gamma globulin, or a mixture of both The results obtained in the anti globulin test will depend to some extent upon the correctness of the dilution of the anti globulin serum employed and whether it contains the antibody for the particular type of globulin with which the cells are coated

As has already been mentioned when blood samples are

anti globulin method, its behaviour and specificity can be investigated (Roth and Frumin, 1957)[18]

When, in the performance of an Indirect anti-globulin test, the serum being investigated appears to react with the antigen on the red cells employed, it should not be confidently assumed that the serum contains antibody until it can also be shown that the test cells are not already coated by a "normal" cold incomplete antibody In practice this hazard is avoided by the setting up of the cell suspension control which is, in effect, a Direct anti globulin test applied to the cells Washed cells which *have not* been exposed to the sensitising serum are subjected to the same dilutions of anti globulin serum at the same time as the main test is performed on the cells which *have* been exposed to the sensitising serum It should then be obvious whether the reaction observed in the main test is due to a coating of normal cold incomplete antibody already present on the red cells, or whether the origin of the antibody now bound to the cells was in the serum undergoing investigation

PLATELETS, LEUCOCYTES AND THE CORRESPONDING ANTIBODIES

The study of blood group antigens in human platelets has always been difficult because of their pronounced tendency to adhere together spontaneously whenever they come into contact with a surface which is not absolutely smooth This viscous metamorphosis, which is part of a physiological mechanism designed to prevent loss of blood from damaged vessels, makes difficult the interpretation of agglutination reactions when studying the antigenic structure of platelets, or when a patient's serum is being examined for the presence of anti platelet antibodies

In 1954 Gurevitch and Nelken[19] described a method for overcoming this spontaneous adhesion of platelets and demonstrated the existence of A and B antigens on their

Hare, Heck and Mathieson (1954),[16] have cast doubt on the value of the tube method of performing the anti-globulin test in the study of hæmolytic anæmia. That the red cells are not alone in being coated by antibody is suggested by the finding of Nelken and Gurevitch (1956)[17] that the patient's platelets may also give a positive Direct anti-globulin reaction.

The Indirect anti-globulin test is used to study the antibody content of the patient's serum. It is advisable to use washed cells of known antigenic structure in order to be able to distinguish an antibody with group specificity from the non-specific type. The serum should be fresh since complement must be available before a cold antibody can manifest its presence by attachment to the red cells. In the initial stages of the investigation, when it is not known whether the antibody is of the cold or the warm variety, tests should be performed both at 20° C. and at 37° C. A particularly avid cold antibody with a wide thermal range may show such activity at 37° C. that the investigator is misled into thinking that a warm antibody is also present. This pitfall can be avoided by heating the washed cells and the patient's serum separately to 37° C. before mixing them together. The capacity of a cold antibody to sensitise cells can often be intensified if the patient's serum is acidified by the addition of N/4 hydrochloric acid (in the proportion of one volume of acid to nine volumes of serum) before subjecting the cells to its action.

The serum of a patient with acquired hæmolytic anæmia very often reacts with all the cells with which it comes in contact, suggesting that a mixture of antibodies is present, some of which are coating the red cells while others are free in the serum. The antibody which will be of most importance to the patient is the one with which his own cells are coated. When this antibody has been eluted off his cells it can be used in the place of a sensitising serum and, by the Indirect

anti globulin method, its behaviour and specificity can be investigated (Roth and Frumin, 1957)[18]

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a papain solution and are then incubated with the serum suspected of containing antibody After a thorough wash to remove unbound antibody the cells are mixed and incubated with a suitably diluted anti globulin serum This mixture is finally added to a suspension of well washed D positive red cells which have been previously coated with anti D antibody If no agglutination now occurs the anti-globulin serum must already have been used up by absorption into combination with an antibody coating the platelets or leucocytes with the result that it appears to be inactive when mixed with red cells which are known to be coated with anti D antibody If agglutination of the sensitised red cells does occur the anti globulin serum has not been "consumed" by the platelets or leucocytes and it can be assumed, therefore that the serum under examination did not contain an antibody specific for the antigens on the platelet or leucocyte surfaces

OTHER APPLICATIONS OF THE ANTI GLOBULIN TEST

Bacteriology

Fifty years ago, in his second paper, Moreschi (1908*b*)[25] described the acceleration and strengthening of bacterial agglutination reactions by what we now call the anti-globulin technique and since its re discovery by Coombs[26] and his colleagues in 1945 many investigators have used this method in studying the agglutination of bacteria As in other fields it has been shown that antibodies directed against bacterial antigens may be of either the complete or incomplete variety or a mixture of both and, furthermore, the titre of the antibody is apparently enhanced when tested by the anti globulin method

Morgan and Schultze (1946)[27] reported the finding in human sera of incomplete antibodies specific for Shigella organisms and anti-Typhoid antibodies could also be

surface if these were present in the red cells In 1955 Coombs and Bedford [20] subjected suspensions of platelets to the action of anti-A or anti B antibody in order to allow absorption of the antibody to the platelet surface When the platelets were washed free from uncombined antibody they were mixed with weak suspensions of A, B or O red cells Platelets from a group-A person, treated with anti A serum, would cause the agglutination of A red cells, but would not cause clumping of B or O cells The mixed platelet erythrocyte agglutination was specific and could only occur providing the platelets and the red cells had antigens in common

If the antibody first taken up by the platelets was of the incomplete type, then, after washing and mixing with the red cells, no agglutination would occur However, Coombs Marks and Bedford (1956) [21] overcame this obstacle by applying the Direct anti globulin method to the platelet erythrocyte mixture In their experimental work they were able to demonstrate anti platelet antibodies produced in rabbits and although in 34 cases of idiopathic thrombo cytopenia in human patients they were unable to prove the existence of anti platelet antibodies the technique has seemed to show sufficient promise to make it worth pursuing

Many workers have demonstrated the occurrence of platelet sensitisation by employment of the Direct anti globulin test In 1956 Jaeger-Draafsel, Weigman and van Loghem [2] used the technique of Dausset and Malinvaud (1954) [3] to search for anti platelet antibodies in the serum of 159 patients and by this method they showed that more than 50 per cent of the patients had an incomplete anti-platelet antibody

The *anti globulin consumption* or *utilisation test* a modification devised by Moulinier (1956) [4] can also be of assistance in the study of antibodies against platelets or leucocytes The platelets or leucocytes are first treated with

cerebro spinal fluid contains small quantities of globulins, but that in certain pathological conditions such as syphilis the amount of globulin is increased

By the *anti globulin inhibition technique* the globulin content of a solution may be investigated Varying strengths of the solution are mixed with serial dilutions of anti globulin serum and are left for at least 30 minutes at room temperature after which each mixture is tested with well washed sensitised cells If agglutination of the sensitised cells occurs the anti globulin serum cannot have been inactivated by globulins in the solution But if no agglutination results the solution must have contained globulins which, by their reaction with the anti globulin serum, appear to inhibit its capacity to agglutinate coated cells

This exposition of the method is correct so far as it goes but the results must be interpreted with circumspection It is possible, for example, that a solution containing non-gamma globulins would neutralise the activity of the anti-non gamma fraction of an anti globulin serum, while leaving the anti gamma globulin moiety free to react with cells coated with gamma globulin antibody, the resultant agglutination would suggest erroneously that the solution had been globulin free Since it is possible to prepare special anti globulin serum which will combine with gamma globulins only, or with non gamma globulins only, it is practicable to measure both the gamma and the non gamma globulins in a solution If still purer anti globulin sera could be prepared the anti globulin inhibition technique might profitably be used to supplement electrophoretic methods of serum analysis (Grubb, 1956) [38]

In table x is shown the pattern of results to be expected when traces of normal serum are mixed with serial dilutions of an anti globulin serum The 'indicator' cells were group O Rh positive red cells coated with incomplete anti-D antibodies (which have been shown to be gamma globulins)

demonstrated by the anti globulin technique The test was applied by Griffiths (1947)[28] to the study of Brucellosis and by Coombs and Stoker (1951)[29] in investigations of Q fever.

Middlebrook and Dubos (1948)[30] devised a modified technique with which to examine sera obtained from patients suffering from tuberculosis Concentrated aqueous extracts of tubercle bacilli were adsorbed on to the surface of red cells in order to produce an agglutinable antigen Admixture of serum from a patient who had proved tuberculosis would usually agglutinate the red cells but occasionally no such reaction was observed By performing an anti globulin test it could be shown that the lack of agglutination was due to the presence of incomplete antibody Those readers who may require more detailed information will be interested in the writings of Hall and Manion (1951),[31] Mynell (1952)[3] and Haberman (1955)[33]

More recently, when using the test to observe the reactions of anti sera against various organisms, Ford and De Falco (1956)[34] concluded, "Such diseases as those caused by animal parasites, allergens, fungi and viruses may possibly be detected with much more facility by applications of the Coombs test or by modifications of this technique We believe that a wider application of this technique in serological tests may be well worth the effort

Estimation of Globulins in Serum and other Fluids

It has been reiterated repeatedly that the successful performance of an anti globulin test depends to a great extent upon the thorough removal by washing of the coated cells of unbound globulin or serum which would neutralise the activity of the anti globulin serum employed This knowledge that small quantities of globulin in solution will inhibit the ability of anti globulin serum to agglutinate coated cells was ingeniously applied by Wiener Hyman and Handman (1949)[35] when they demonstrated that normal

In contrast, tables XI and XII illustrate the very different disposition of reactions given by sera from two patients. The patient who suffered from multiple myeloma had the characteristic hyperglobulinæmia associated with this disease so that even in high dilution his serum inhibited the anti-globulin serum, whereas the serum from a small child afflicted by congenital agammaglobulinæmia had very little neutralising effect. It follows, therefore, that the anti globulin inhibition test can be used in the diagnosis and in the control of treatment of agammaglobulinæmia. The results of this test can be obtained more quickly than can those of biochemical methods and Muralt, Barandun and Hassig (1957)[37] claim that the test will detect gamma globulins in concentrations too low to be detected by electrophoresis.

Forensic Medicine

Just as fluid human serum will inhibit the action of anti human globulin serum, so also will dried human sera or blood stains. Allison and Morton (1953),[38] using the anti-human globulin inhibition method, claimed that it could identify with absolute specificity dried blood or serum stains of human origin which were ten years old, and that it worked successfully with mummified remains at least 5000 years old. Anderson (1954)[39] while agreeing that the test was more specific than the standard anti-human precipitin test, doubted whether it was so sensitive. He found that chimpanzee blood and serum were as efficient as human blood in inhibiting the action of anti human globulin serum though Rhesus monkey serum had no effect. It would be interesting to discover whether a chimpanzee anti human globulin serum could be prepared which might prove to be a more specific reagent.

The first step in the identification of stains is to cut out a small piece of the stained material and to take also an unstained piece of the same size from the same material,

TABLE X

Serial Dilutions AHG 65	Serial Dilutions—Anti A 758 1 100	200	400	800	1600	3200	6400	12800	25600	51200	Sensitised cells	Non sensitised cells
1 2	−	−	+	+	+	+	+	+	+	+	+	+
4	−	−	+	+	+	+	+	+	+	+	+	−
8	−	−	−	−	+	+	+	+	+	+	+	−
16	−	−	−	−	−	+	+	+	+	+	+	−
32	−	−	−	−	−	−	+	+	+	+	+	−
64	−	−	−	−	−	−	−	−	−	−	+	−
128	−	−	−	−	−	−	−	−	−	−	+	−
256	−	−	−	−	−	−	−	−	−	−	+	−
512	−	−	−	−	−	−	−	−	−	−	−	−

which will serve as a control The two pieces are placed in separate but equal, amounts of the same standard anti human globulin serum and are left overnight On the following day the test fluid and the control fluid are each titrated separately against both sensitised and non sensitised red cells The cells used are human group O Rh positive erythrocytes, one portion of which has been coated with incomplete anti D antibody while the other portion has been left untreated The point of testing the control fluid is to show whether the material alone has any inhibitory effect on the anti globulin serum When the test fluid is mixed with the sensitised cells and no agglutination results, provided that the control tests have been satisfactory, the stain can be assumed to be of human origin

Hæmolytic Disease of the Newborn in Animals

Consequent upon the discovery of the underlying cause of hæmolytic disease of the newborn in human babies, it was natural to turn attention to the young of animals and to try to find out whether any of the neonatal deaths occurring in other species could be attributed to similar blood group incompatibility mechanisms It was soon established that hæmolytic disease of the newborn could occur in horses (Coombs, Crowhurst, Day, Heard, Hinde, Hoogstraten and Parry, 1948 [40]), in mules (Caroli and Bessis, 1947 [41]), and in pigs (Buxton Brooksbank and Coombs, 1955 [4]) and it has been experimentally produced in animals of other species too

Cronin (1955) [43] considers that 1 per cent of thorough bred racehorses in the Newmarket area have foals afflicted by hæmolytic disease of the newborn and Brion (1949) [44] estimated that in the mule breeding region of France the incidence in mule foals is as high as 8 per cent The incidence in pigs has not yet been published, but even if the frequency is only 0 5 per cent then the loss from the disease in these three species of animals is of economic importance

TABLE XI

Serial Dilutions—H S Serum—Multiple Myeloma

Serial Dilutions AHG 65	1 100	200	400	800	1600	3200	6400	12800	25600	51200
1 2	–	–	–	–	+	+	+	+	+	+
4	–	–	–	–	–	+	+	+	+	+
8	–	–	–	–	–	–	–	+	+	+
16	–	–	–	–	–	–	–	–	–	–
32	–	–	–	–	–	–	–	–	–	–
64	–	–	–	–	–	–	–	–	–	–
128	–	–	–	–	–	–	–	–	–	–
256	–	–	–	–	–	–	–	–	–	–
512	–	–	–	–	–	–	–	–	–	–

TABLE XII

Serial Dilutions—E J M Serum—Agammaglobulinæmia

Serial Dilutions AHG 61	1 100	200	400	800	1600	3200	6400	12800	25600	51200
1 1	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+	+	+
32	+	+	+	+	+	+	+	+	+	+
64	–	+	+	+	+	+	+	+	+	+
128	–	+	+	+	+	+	+	+	+	+
256	–	–	–	–	–	+	+	+	+	+
512	–	–	–	–	–	+	+	+	+	+

by a foster mother whose milk does not contain the noxious antibodies

The Indirect anti globulin test has been used to search for antibodies in the sera of mares and sows and it has been possible to demonstrate in these sera both complete and incomplete antibodies which will react with the red cells of their mates. Further studies of the blood groups of horses and pigs should enable breeders to select suitable sires for their animals, thus avoiding the intervention of hæmolytic disease with its consequent economic losses

In the period 1948-1952 an excellent series of papers was published by Young and his colleagues, who chose dogs as the animals in which to study experimentally produced hæmolytic disease and various transfusion problems. The logical first approach was to delineate the canine blood-group systems. Among other interesting observations, Christian, Ervin and Young (1951)[45] demonstrated that dogs may have an antigen, A^1, with serological characteristics which are very similar to those of the D^u antigen in man. Like D^u, the A^1 antigen is readily detected by the action of dog anti-A serum in the Indirect anti globulin test. Swisher and Young (1954)[46] were able to prove by the same method that canine antibodies may exist in the incomplete form. Hæmolytic disease of the newborn was experimentally produced in pups and, although at birth the Direct anti-globulin test applied to the pups red cells gave a negative result, the reaction became positive when they were suckled by their mother (Young, Christian, Ervin, Davies, O Brien, Swisher and Yuile, 1951)[47]

Studies of blood groups and experimental hæmolytic disease of the newborn in rabbits have shown that the anti globulin technique is an exceedingly useful tool with which to detect incomplete antibodies and to demonstrate sensitisation of cells. Heard, Hinde and Mynors (1949),[48] Kellner and Hedal (1952),[49] and Anderson (1956)[50]

The ætiology of the disease in animals differs slightly from the mechanism producing human hæmolytic disease of the newborn The placentæ of most animals have several chorio-epithelial layers which provide a greater barrier to the passage of antibodies from mother to fœtus than does the single layer of the human placenta As a result, the offspring are born healthy, having been unaffected *in utero* by maternal antibody, but they succumb a few days later to the effects of maternal antibody secreted in the colostrum and mother's milk

The blood-group systems of horses, donkeys and pigs have not been studied to the same extent as those in man and knowledge about the behaviour of the antigens and antibodies involved is less complete Just as in man, the discovery in the laboratory of serological differences in the blood groups of mother and offspring does not mean necessarily that incompatibility exists which will have clinical repercussions Before making a diagnosis of hæmolytic disease it is necessary to show that antibodies directed against antigens on the red cells of the fœtus are present in the maternal serum and in the colostrum and that these antibodies are able to coat or destroy the red cells of the young animal Confirmation that hæmolytic disease of the newborn is the underlying cause of some cases of anæmia in sucklings is provided by the finding that the red cells, which give a negative Direct anti globulin reaction at birth subsequently show evidence of sensitisation in a positive Direct anti globulin result after the ingestion of colostrum

For the performance of such tests special anti globulin sera must be prepared In the study of the disease in pigs rabbit anti pig globulin serum is used in investigations involving horses and mules rabbit anti horse globulin serum is the appropriate reagent

Once the diagnosis has been made the anæmia may be cured by transfusion or the young animals may be suckled

against the conjugate. There may be many other applications of this test in the study of proteins, polypeptides and polysaccharides of biological and pathological importance. It has great possibilities in the sphere of allergy.

A problem of blood group serology which still awaits full investigation is the determination of the number of antigen receptors, pertaining to each blood group system on the red cells. The use of a radioactive iodine method led Boursnell, Coombs and Rizk (1953)[54] to postulate that 5500 Rh (D) antigen receptors were available on each red cell. Wiener and Gordon (1953)[9] described a quantitative anti globulin test which they considered could be applied to determine the relative number of antigen receptors on the red cells for the various blood group systems. The same problem was tackled by Grubb (1955),[55] using the anti-globulin technique in a rather different way. Suspensions of well washed Rh positive red cells were prepared in volumes known to contain about 10^9 cells. To each volume of cells was added a measured volume of anti-D serum in various dilutions and the mixtures were then incubated to allow the cells to become coated. After thorough washing to remove free antibody, measured volumes of diluted anti globulin serum were added to the sedimented cells. At the same time identical dilutions of anti globulin serum were mixed also with standard solutions of gamma globulin. A comparison was then made of the ability to agglutinate further amounts of coated cells by the anti globulin solution neutralised by red cells and by the anti globulin solution inhibited by standard gamma globulin. From this could be found the amount of gamma globulin in a particular dilution of anti D antibody which combined with a known number of red cells and, assuming that the molecular weight of gamma globulins is 165,000, Grubb calculated that there must be a minimum of 2000 receptors on each red cell.

In other species of animal, cattle, for instance, the blood group systems have been intensively investigated, but much remains to be discovered and the veterinary scientist will find the anti-globulin technique a necessity for a complete study

Immunological Research

Coombs, Gleeson White and Hall (1951)[51] devised a *multi-stage anti globulin technique*, building up an anti globulin globulin lattice in order to effect agglutination of certain bovine red cells which had been sensitised by heterophile antibody, but which were not agglutinated by the ordinary single stage anti globulin test (Gleeson White, Heard, Mynors and Coombs, 1950)[52] These two papers made it clear that, in at least one species although the red cells may possess a particular antigen, it will only be detected by superimposing on the cells specifically combined alternate layers of antibody globulin anti-globulin antibody globulin and anti globulin until agglutination occurs

It has been recognised for many years that the agglutina tion reaction provides a much more sensitive method than precipitation for demonstrating the specific antibody content of serum When the antigen is a soluble protein and the precipitin antibody is of the incomplete type any interaction which occurs between them will not be readily detectable Coombs, Howard and Wild (1952),[53] attempting to find a way in which to recognise that such reactions had taken place contrived a sensitive agglutination method in preference to precipitation They were able to conjugate the soluble protein antigen with an incomplete anti D antibody and then, by mixing the conjugate with Rh positive cells to coat those cells with anti D antibody conjugated with protein antigen This coating could be detected either by testing the red cells with anti human globulin serum or by the use of a specially prepared anti globulin serum active

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CHAPTER XI

ODDMENTS

THIS book is not intended to be a complete review of the anti globulin test, but rather to act as a broad basis on which the student can build It is for this reason that, in this chapter, a few oddments are mentioned which may suggest lines on which future work could be planned

THE ANTI GLOBULIN TEST IN CANCER

Green, Wakefield and Littlewood (1957),[1] using anti human globulin serum at lower dilutions than standard (i e at dilutions which were more likely to pick up non gamma than gamma globulin antibodies), found that the red cells of 43 per cent of patients with cancer gave a positive Direct anti globulin reaction whereas in non cancerous patients the Direct anti globulin test was positive in 15 per cent The degree of positivity was stated to be related to tumour size and was greater with larger tumour masses Tumours of lymphoid tissue and advanced carcinoma gave the greatest number of positives These authors suggested that the anæmia of malignant disease might be due to an auto antibody produced by the tumour cells and from the evidence presented, the antibody they were detecting was of the non gamma globulin type

Dunsford (1957) found that the sera of patients with cancer more often contained auto agglutinins or pan agglutinins than did the sera of patients who did not have cancer It was an antibody of this type which caused the positive Direct anti globulin test in a case of pernicious anæmia reported by Selwyn and Alexander (1951)[3] and it may have been this sort of antibody which was responsible for the findings of Green[1] and co workers In a small

series of 40 cases Dunsford[2] observed that the Direct anti globulin test was always negative if the cells from patients with malignant disease were tested before they had been allowed to cool to a temperature below 30° C Madden (1956)[4] reported positive Direct anti globulin reactions given by sera obtained from patients suffering from cancer if the samples had been first refrigerated

Auto antibodies appear to play a definite role in acquired hæmolytic anæmia and in Hashimoto s and other diseases (Roitt, Doniach, Campbell and Vaughan-Hudson, 1956)[5] Furthermore, normal healthy people can be shown to have anti H as a normal incomplete cold antibody in their sera—Crawford, Cutbush and Mollison (1953)[6]—and yet at the same time these people have H-substances in their tissues One possible hypothesis advanced suggests that if this auto tolerance should break down in a particular organ the tissue cells may become so modified that they lose their tissue specificity and result in a malignant condition Certainly the theory of the immunological basis of cancer enunciated by Green[1] and his colleagues and the observations recorded by them warrant further investigation

The Anti Globulin Test in Lead Poisoning

Sutherland and Eisentraut (1956),[7] using the tube method of performing the anti globulin test advocated by Rosenfield, Vogel and Rosenthal (1951)[8] found that in the anæmia produced by lead intoxication in humans and in experimentally induced lead poisoning in dogs, the red cells gave a positive Direct anti globulin reaction

Dunsford (unpublished) also has observed a strong positive Direct anti globulin result given by the red cells of a child suffering from severe lead poisoning although negative reactions were obtained in tests on the bloods of three men, workers in the car industry, who had been exposed

to lead for some time and all of whom had marked symptoms of plumbism These interesting observations merit further *investigation by those making a special study of lead poisoning*

The Anti Globulin Test and Other Chemicals

Following the intravenous administration of phenyl hydrazine to a dog, Muirhead, Groves and Bryan (1954*a*) [9] found that both the Direct and Indirect anti globulin reactions became positive These positive results could not be obtained if phenylhydrazine was added to the dog's blood after collection, in other words, the phenylhydrazine was not able *in vitro*, to modify the dog s red cells so that they became agglutinable by anti globulin sera The same team Muirhead, Groves and Bryan (1954*b*) [10] however reported later that the addition of hydrazine to the dog blood *in vitro* simulated the phenomena observed after in vivo action of phenylhydrazine The authors considered several possible explanations It might be that hydrazine uncovered protein on the dog erythrocytes which would react with the rabbit anti dog antibodies to produce a positive Direct anti globulin result, perhaps the hydrazine altered the surface of the dog erythrocytes in such a way that it absorbed proteins, or it might be that the hydrazine acted as a coupling agent between the red cells and the normal proteins, Muirhead and Groves (1956) [11] further reported that lipid solvents acting *in vivo* modified dog erythrocytes so that they gave a positive Direct anti globulin reaction

As reported by Jandl (1955) [1] and Jandl and Simmons (1957) [13] several metallic cations are capable of attaching proteins to the surfaces of human red cells, thus sensitising" the cells, which can then be agglutinated by anti sera specific for the proteins attachment For instance, anti human globulin serum agglutinated the cells if human

globulin had been attached to the red cells by the action of metallic cations The effect was observed when the cations Cr^{+++}, Fe^{+++}, Be^{+++}, Rh^{+++}, and Al^{+++} were employed Red cells so treated can be used as positive controls for the anti-globulin test Such cells will keep for at least a week

A Test which may Supplement the Anti Globulin Technique

When a 1 per cent solution of protamine sulphate was added to a saline suspension of red cells coated with incomplete anti D antibodies agglutination occurred (Roth and Frumin, 1954)[14] Cells which had not been coated by antibody were not agglutinated and neither were cells obtained from cases of acquired hæmolytic anæmia After further observations Roth and Frumin (1957),[15] described the results obtained with this and other tests on red cells from 17 patients suffering from acquired hæmolytic anæmia In some cases agglutination occurred, in other cases it did not This may have been due to a distinction in the globulin coating of the red cells, such as the difference between gamma and non gamma globulins More study is necessary for the understanding of the mechanism and for an evaluation of the helpfulness of this method as a supplementary technique in the investigation of hæmolytic disease

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